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Identification of the chemokine CCL28 as a growth and survival factor for human hematopoietic stem- and progenitor cells

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Running title

CCL28 stimulates primitive hematopoietic cells

Key points

1. Chemokine (C-C motif) ligand 28 (CCL28) is a novel growth factor for human hematopoietic stem- and progenitor cells.
2. CCL28 supports the *in vitro* and *in vivo* functional integrity of cultured primitive hematopoietic cells.

Abstract

In an attempt to discover novel growth factors for hematopoietic stem- and progenitor cells (HSPCs), we have assessed cytokine responses of cord blood (CB)-derived CD34⁺ cells in a high-content growth factor screen. We identify the immunoregulatory chemokine (C-C motif) ligand 28 (CCL28) as a novel growth factor that directly stimulates proliferation of primitive hematopoietic cells from different ontogenetic origins. CCL28 enhances the functional progenitor cell content of cultured cells by stimulating cell cycling and induces gene expression changes associated with survival. Importantly, addition of CCL28 to cultures of purified putative hematopoietic stem cells (HSCs) significantly increases the ability of the cells to long-term repopulate immunodeficient mice compared to equivalent input numbers of fresh cells. Together, our findings identify CCL28 as a potent growth-promoting factor with the ability to support the *in vitro* and *in vivo* functional properties of cultured human hematopoietic cells.

Introduction

Extrinsic signaling molecules have been widely examined for their potential to support hematopoietic stem cells (HSCs) *ex vivo*. However, few growth factors have been identified that maintain the primitive properties of HSCs.^{1, 2} Recently, several proteins or small molecules were shown to increase the numbers of cultured human hematopoietic stem- and progenitor cells (HSPCs), but the majority of these rely on the basic support of at least three different cytokines,³⁻⁸ some of which may promote differentiation at the expense of HSC maintenance.^{1, 2, 9} Thus, current culture conditions for HSPCs should be improved by novel HSC-supportive factors. Using a systematic screening approach, we have identified chemokine (C-C motif) ligand 28 (CCL28) as a promising new growth factor that preserves the functional integrity of human HSPCs.

Methods

Culture conditions and progenitor cell assays

HSPCs were cultured in Serum-Free Expansion Medium (StemCell Technologies) supplemented with stem cell factor (SCF) at 10 ng/mL (“S10”) or 100 ng/mL (“S100”). For screening (n=276) and validation (n=36), recombinant cytokines (Peprotech) were used at 100 ng/mL. Colony forming cell (CFC) assays were established according to manufacturer’s instructions (StemCell Technologies). Long-term culture-initiating cell (LTC-IC) assays are described elsewhere.¹⁰ For details, see Supplemental Methods. Human cord blood (CB) was collected from umbilical cords at the end of full-term deliveries obtained from the Department of Obstetrics in Lund, Sweden, with the informed consent of the mothers, or purchased from the Anthony Nolan Cell Therapy Centre, London, UK. Human bone marrow (BM) aspirates were obtained from the posterior iliac crest of fully informed healthy donors from the Department of Hematology in Lund. This study was conducted in accordance with the Declaration of Helsinki.

Microarray (GEO accession number: GSE45136), cell cycle and apoptosis assays

See Supplemental Methods.

Xenotransplantation

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ mice (NSG; Jackson Laboratory) were used for animal experiments, as approved by the Lund/Malmö Ethical Committee. For details, see Supplemental Methods.

Statistical analyses

Statistical significance was calculated using paired t-test. Unless otherwise stated, error bars indicate standard error of the mean (SEM).

Results and discussion

To discover novel growth factors for HSPCs, we designed a screening assay for expansion of CB-derived CD34⁺ cells based on high-throughput FACS analysis of CD34 expression following a 7-day culture period. Given the high proliferative potential of CB progenitors,¹¹⁻¹³ we hypothesized that the power of each factor would be best distinguished in low concentrations of SCF, which promotes survival without provoking extensive proliferation.^{14, 15} A concentration of 10 ng/mL SCF was sufficient to maintain survival and induce strong synergistic effects on CD34⁺ cell expansion together with the known HSC-supportive factor thrombopoietin (TPO)¹⁶ (Figure S1A). Thus, we used this condition to systematically screen 276 human growth factors for their potential to expand CD34⁺ cells (Figure 1A). Based on two independent screens (Table S1E) and three consecutive validation experiments (Figure 1B and S1B), CCL28 showed the most prominent and consistent expansion of CD34⁺ cells, and was therefore selected for further investigation.

To determine the contexts in which CCL28 stimulates HSPCs, we first evaluated the effect of increasing CCL28 doses at different SCF concentrations. We observed a dose-dependent increase in CD34⁺ cell numbers in response to stimulation with 50-1000 ng/mL CCL28 (Figure 1C and S3B). At 500 ng/mL the effect of CCL28 reached saturation and yielded similar synergistic effects on CD34⁺ cell expansion as TPO (Figure 1C and S2). Bone marrow (BM)- and fetal liver (FL)-derived CD34⁺ cells were stimulated in a similar manner (Figure 1D). CFC assays showed that CCL28, similarly to TPO, induced a robust expansion of functional progenitor cells together with SCF (Figure 1E and S3A). CCL28 also promoted growth, but to a lesser extent, in combination with TPO or fms-like tyrosine kinase 3 ligand (FLT3L) (Figure S3C-D). When added alone, CCL28 failed to induce a net proliferation of CD34⁺ cells, but was sufficient to maintain progenitor activity (Figure 1F-G), suggesting that it provides crucial survival signals for primitive hematopoietic cells.

To understand the basis of the cellular response to CCL28 stimulation, we performed transcriptional profiling and gene set enrichment analysis (GSEA).¹⁷ Treatment with either CCL28 (Figure 1H) or TPO (Figure S4) caused a significant enrichment of genes correlating with the GO term cell cycle. Genes associated with cell viability were significantly enriched in CCL28-stimulated cells, but not in cells treated with

TPO (Figure 1H and S4). The gene expression signatures translated into effects on cell cycling with higher frequencies of dividing cells following CCL28 and TPO stimulation (Figure 1I and K). Moreover, we observed a trend towards decreased apoptosis in CCL28 stimulated cells (Figure 1J and L). Thus, CCL28 supports proliferation of hematopoietic progenitors by stimulating cell cycling and by suppressing apoptosis.

CCL28 signals through the two cell surface receptors CCR3 and CCR10.^{18, 19} We detected CCR10, but not CCR3, expression in the vast majority of CD34⁺ cells and in the HSC-enriched CD34⁺CD38⁻ population (Figure 2A), suggesting that CCL28 operates through CCR10 on the earliest hematopoietic progenitors. To assess whether CCL28 indeed supports the most immature fraction of HSPCs, we performed LTC-IC assays, which showed preserved LTC-IC activity of CCL28-treated cultures, as compared to starting equivalents of fresh cells (Figure 2B-C). These results prompted us to investigate the impact of CCL28 treatment on growth and engraftment capacity of CB-derived putative human HSCs (CD34^{hi}CD38^{lo}CD90⁺CD45RA⁻ cells).²⁰ As indicated by receptor expression, CCL28 stimulated putative HSCs in a direct manner, demonstrated by increased cell numbers and progenitor activity compared to SCF-treated cultures (Figure 2D-G). While TPO treatment outranged CCL28 stimulation with regard to overall proliferation potential, CCL28 maintained a higher frequency of the most primitive cell fractions (Figure 2F). Additionally, transplantation of CCL28-stimulated HSCs into NSG mice²¹ yielded 62% engrafted recipients four weeks post transplant, whereas only 9% and 36% of the mice transplanted with SCF- and TPO-treated cells, respectively, displayed human chimerism above 1% (Figure 2H and table S2). Compared to equivalent numbers of fresh cells, CCL28-treated cells showed slightly lower short-term engraftment, indicating either a subtle loss of short-term HSCs or delayed engraftment kinetics of the cultured cells (Figure S5). By contrast, CCL28 treatment significantly improved long-term multilineage reconstitution levels compared to both fresh cells and cells cultured with SCF alone, demonstrating that the combined effect of SCF and CCL28 supports a net increase of long-term repopulating activity (Figure 2I-K). TPO-treated cells showed similar engraftment levels as CCL28, although not significantly increased compared to fresh cells. Collectively, these findings implicate CCL28 as a new, potent growth factor that supports the functional integrity of *ex vivo*-cultured HSPCs, placing it in a unique category of cytokines with HSC-supportive functions such as SCF and TPO.

To assess how CCL28 affects HSPCs in the context of a rich cytokine cocktail, we added it in combination with SCF, TPO and FLT3L (“STF”) to cultures of CB CD34⁺ cells. STF conditions, in general, supported CFCs and led to elevated numbers of LTC-ICs compared to fresh cells, while NSG engraftment levels were markedly reduced (Figure S6), indicating a strong proliferative drive but poor maintenance of the most primitive cells. Addition of CCL28 to these conditions enhanced the output of early progenitors with mixed CFC potential, but did not rescue the adverse effects of STF stimulation on NSG reconstitution ability (Figure S6). These findings illustrate the context-dependent function of growth factors and support the notion that multiple cytokine stimulation promotes proliferation at the expense of HSC activity.^{9, 22} Thus, the further assessment of CCL28 to improve *ex vivo* culture conditions for HSPCs should be conducted in a systematic manner with other growth factor contexts and/or recently discovered molecules for HSC expansion.^{3-6, 10, 23} Finally, given the presence of CCL28 in the BM microenvironment²⁴ (Figure S7), the potential role of CCL28 in regulating HSPCs *in vivo* as a niche-secreted factor under both normal and malignant²⁵ conditions represents another interesting topic for future studies.

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Authorship

Contribution: J.L. and C.K. designed the study; C.K., A.B., N.M., G.K., and R.G. performed research; S.S. and M.M. performed microarray data analysis. T.E. provided scientific expertise. C.K. and J.L. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure legends

Figure 1. A high-content growth factor screen in human CB progenitors identifies CCL28 as potent stimulator of HSPC proliferation.

(A) Outcome of the primary screen. Proliferation of cells cultured in S10 was set to 1 and relative fold increase of CD34⁺ cells was determined after 7 days of culture. The mean values from 2 screens are shown. The experimental design of the primary screen is shown as an overlay. (B) Outcome of the validation. Plotted are screening and validation results from 36 selected candidate factors, $n = 4$. Shown are floating bars from the minimum to the maximum, the line indicates the mean. (C) CB CD34⁺ cells were cultured in S10 or S100 together with increasing concentrations of TPO or CCL28, and analyzed for proliferation and CD34 expression by FACS at day 7; $n = 3-4$. Following titration, CCL28 was used at 500 ng/mL. (D) FL- and BM-derived CD34⁺ cells were cultured in S10 and analyzed for proliferation and CD34 expression by FACS at day 7; $n = 2-3$, error bars indicate standard deviation (SD). (E) Numbers of total CFCs per an equivalent of 100 input CB CD34⁺ cells at day 0; $n = 3$. (F-G) Proliferation (F; $n = 7$) and CFC (G; $n = 5$) potential of CB progenitors cultured in single cytokine stimulation for 7 days. (H) GSEA results for cell cycle and cell death signatures in SCF vs. SCF + CCL28 conditions. NES, normalized enrichment score; NOM P, nominal P -value; FDR, false discovery rate. (I-J) Representative cell cycle (I) and apoptosis (J) FACS plots of CD34⁺-gated cells. (K-L) Quantification of cell cycle (K) and apoptosis (L) data, $n = 5$. The dashed lines in figures (C, D and F) indicate input cell numbers; black and grey bars represent CD34⁺ and CD34⁻ cells, respectively. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.01$; ns = not significant, na = not applicable.

Figure 2. CCL28 directly stimulates putative human HSCs and improves the long-term repopulating activity.

(A) Representative histogram plots for cell surface expression of CCR3 and CCR10 in different fractions of CB ($n = 5$). FMO, fluorescence minus one; MNC, mononuclear cells. (B-C) CD34-enriched CB cells were plated in limiting dilutions either at day 0 or day 7 after culture in S10 and the indicated cytokines. Shown are LTC-IC numbers (B) and frequencies (C) as calculated by L-Calc; $n = 3$. (D-F) CB CD34^{hi}CD38^{lo}CD90⁺CD45RA⁻ cells were

cultured in S10 and the respective cytokines and monitored for proliferation and progenitor activity. (D) Representative bright-field microscopy images (i) and FACS plots (ii) of the progeny from 1×10^2 CD34^{hi}CD38^{lo}CD90⁺CD45RA⁻ cells at day 10. (E-F) Total cell number (E) and frequency (F) of CD34⁻, CD34⁺CD90⁻ and CD34⁺CD90⁺ cells as determined by FACS analysis at day 10-13; n = 3. (G) Numbers of differential CFCs per an equivalent of 100 input CD34^{hi}CD38^{lo}CD90⁺CD45RA⁻ cells at day 0; n = 4, (statistical significance was calculated on total CFCs). Delta (Δ) indicates the difference between TPO- and CCL28 treatment. (H-K) CD34^{hi}CD38^{lo}CD90⁺CD45RA⁻ cells were transplanted either directly or following 7 days of culture with the indicated cytokines into sublethally irradiated NSG recipients. Engraftment levels of human cells in peripheral blood (PB) after one (H) and four (I) months are shown. The dashed line in (H) marks the 1% cut-off for positive engraftment. Each data point represents an individual mouse, shown is data from three independent experiments with n = 3-5 recipients/group. Experiment 1: 1×10^3 IEM, experiment 2 and 3: 2×10^3 IEM; IEM, input equivalents/mouse. (J) Collated data showing recipient means across all experiments. (K) Lineage distribution in PB of NSG recipients four months post transplantation. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 1

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