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Long-Acting IL-33 Mobilizes High-Quality Hematopoietic Stem and Progenitor Cells More Efficiently Than Granulocyte Colony-Stimulating Factor or AMD3100

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Mobilization of hematopoietic stem and progenitor cells (HSPCs) has become increasingly important for hematopoietic cell transplantation. Current mobilization approaches are insufficient because they fail to mobilize sufficient numbers of cells in a significant fraction of patients and are biased toward myeloid immune reconstitution. A novel, single drug mobilization agent that allows a more balanced (myeloid and lymphoid) reconstitution would therefore be highly favorable to improve transplantation outcome. In this present study, we tested commercially available IL-33 molecules and engineered novel variants of IL-33. These molecules were tested in cell-based assays in vitro and in mobilization models in vivo. We observed for the first time that IL-33 treatment in mice mobilized HSPCs and common myeloid progenitors more efficiently than clinical mobilizing agents granulocyte colony-stimulating factor (G-CSF) or AMD3100. We engineered several oxidation-resistant IL-33 variants with equal or better in vitro activity. In vivo, these variants mobilized HSPCs and, interestingly, also hematopoietic stem cells, common lymphoid progenitor cells, and endothelial progenitor cells more efficiently than wild-type IL-33 or G-CSF. We then engineered an IL-33-Fc fusion molecule, a single dose of which was sufficient to significantly increase the mobilization of HSPCs after 4 days. In conclusion, our findings suggest that long-acting, oxidation-resistant IL-33 may be a novel approach for HSPC transplantation. IL-33-mobilized HSPCs differ from cells mobilized with G-CSF and AMD3100, and it is possible that these differences may result in better transplantation outcomes.

INTRODUCTION

Standard treatment for hematopoietic stem and progenitor cell (HSPC) mobilization is granulocyte colony-stimulating factor (G-CSF) despite the fact that it fails to mobilize sufficient numbers of HSPCs to ensure successful engraftment in up to about one-third of patients [1,2]. Risk groups for poor mobilization can be identified by age, confounding diseases, and previous chemoradiation or irradiation therapy [3]. In these risk groups, additional mobilization factors are considered, most prominently the CXCR4 antagonist AMD3100, also known as plerixafor [4,5]. Although AMD3100 can increase the number of mobilized HSPCs, it cannot overcome the myeloid-biased bone marrow reconstitution [6]. Novel treatments are therefore urgently needed to improve HSPC mobilization strategies for bone marrow transplantation.

IL-33 is a protein for which 2 interesting functions have been described: IL-33 can act as an intracellular transcription factor/regulator or as a local or systemic cytokine following its enzymatic activation and release from the cells [7,8]. The receptor for IL-33 consists of the IL-33-specific receptor molecule IL-1 receptor-like 1 (IL1RL1; also known as ST2, ST2L, ST2V, IL-33R, FIT-1, DER4) and the IL-1 receptor accessory protein (IL1RAP) [9]. While IL1RL1 is specific for IL-33, IL1RAP is shared between IL-1 family receptors. Upon binding of IL-33 to the extracellular domain of IL1RL1, the IL-33-IL1RL1 complex adopts to a conformation that allows recruitment of the accessory receptor molecule IL1RAP, leading to ternary complex formation and initiation of downstream signaling events, starting with the activation of the intracellular Toll/interleukin-1 receptor (TIR) domains [10,11]. Interestingly, upon release

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from the cells, cysteine residues within IL-33 can undergo oxidation, leading to the formation of intramolecular disulfide bridges and a conformational change that disables IL-33 from binding to IL1RL1 and therefore renders IL-33 inactive [12,13].

Although initial findings and a large number of studies associate IL-33 with T helper 2 cytokine responses [7,14], IL-33 also has been described to ameliorate or exacerbate a wide range of inflammatory, infectious, neurologic, and cardiovascular diseases [9,15,16]. Most interestingly, IL-33 induced mobilization of myeloid and B cell progenitor cells that are capable of reconstituting the bone marrow of irradiated mice [17]. This effect was MyD88 dependent, suggesting that IL-33 acts through IL1RL1 [17].

On the basis of these observations, we hypothesized that triggering the IL1RL1 receptor with long-acting IL-33 variants may represent a novel approach to more efficiently mobilize HSPCs of a higher quality than the current standard of care for bone marrow transplantation. To test this hypothesis, we generated novel human IL-33 variants and tested them as well as wild-type IL-33 in comparison to G-CSF and AMD3100.

MATERIALS AND METHODS

IL-33 Reporter Assay

A cell-based secreted embryonic alkaline phosphatase IL-33 reporter assay was used according to the manufacturer's instructions (Invivogen, San Diego, CA). In brief, reporter cells were incubated in the presence of test articles for 24 hours. Quanti-Blue reagent was added, and absorbance was measured at 620 nM.

Human umbilical vein endothelial cell (HUVEC)-CCL7 Secretion Assay

Human umbilical vein endothelial cells (HUVECs) were incubated in the presence of IL-33 variants. Supernatants were collected, and CCL7 secretion was measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Mobilization In Vivo Studies

Male 6- to 10-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Sacramento, CA) and used for the experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Bayer, US Innovation Center, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were treated for up to 4 days with IL-33 variants or G-CSF or once with AMD3100, and heparinized blood was collected and analyzed by flow cytometry or colony forming unit (CFU) assays.

Flow Cytometry

Cells were stained for flow cytometry analysis in a 1-step staining procedure as previously described [18]. In brief, red blood cells were lysed and cells were washed with a flow cytometry buffer (PBS with 5% calf serum and 0.05% sodium azide). Fixed numbers of polystyrene beads (Polysciences, Warrington, PA) were added to each sample for quantitative analysis. Cells were stained with the directly conjugated antibodies with suitable fluorophores, incubated on ice for 20 minutes, and washed. 7-Aminoactinomycin D (BD, San Jose, CA) was used as a viability stain, and cells were measured with an LSR II (BD).

To measure IL1RL1 surface expression, HUVECs were detached using 5 mM EDTA in PBS for 45 minutes and then stained with a directly conjugated IL1RL1 antibody using a 1-step procedure as described above.

Data were analyzed with FCS Express (De Novo Software, Los Angeles, CA).

Colony-Forming Assay

Hematopoietic cells from spleen or peripheral blood were collected and suspended in Methocult with mouse growth factors (GF M3434; Stem Cell Technologies, Vancouver, Canada) at a final concentration of 100,000 to 300,000 cells/mL and cultured in 6-well dishes for 12 days according to the manufacturer's instructions. Colonies were scored and quantified using a STEMVision imaging instrument (Stem Cell Technologies).

Structure Analysis and Visualization

Structural models of IL-33-ST2-IL1RAP complex were generated by overlaying of IL-33-ST2 structural complex (protein data bank code: 4KC3) with IL1β-IL1R1-IL1RAP structural complex (protein data bank code: 4DEP) in Molecular Operating Environment, 2013.08 (Chemical Computing Group ULC, Montreal, Quebec, Canada). Subsequent structural analysis and image creation were done in Molecular Operating Environment and UCSF Chimera [19].

Statistical Analysis

Statistical calculations were performed using Prism 7.0 (GraphPad Software, San Diego, CA).

Additional Methods

Additional details regarding materials, protein expression, purification, and deglycosylation can be found in the supplemental data.

RESULTS

IL-33 Mobilizes HSPCs More Efficiently Than G-CSF or AMD3100

To evaluate the potential of IL-33 to mobilize HSPCs alone and in combination with clinical mobilizing agents G-CSF and AMD3100, mice were treated once daily for 3 days with IL-33 i.p., G-CSF s.c. (the clinical dosing route), or a single dose of AMD3100 i.p. 1 hour before blood collection on day 4. Following treatment with IL-33 alone, we observed mobilization of HSPCs characterized by Lin$^-$/SCA1$^+$/c-Kit$^+$ (LSK) surface marker expression, whereas G-CSF or AMD3100 alone showed only minimal or no effect (Figure 1A and B). When evaluating the

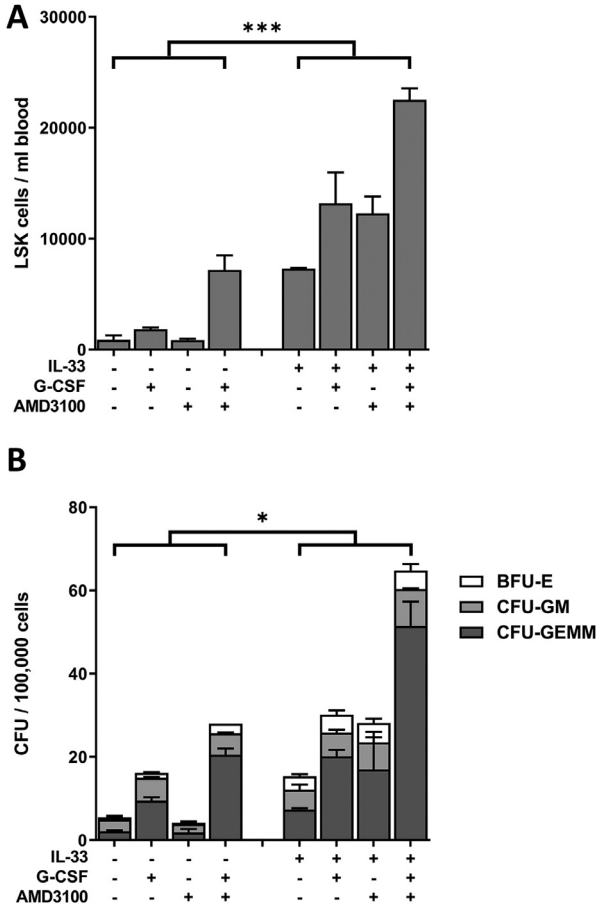

Figure 1. IL-33 mobilizes hematopoietic stem and progenitor cells more efficiently than G-CSF or AMD3100. Mice were treated with murine IL-33 (0.04 mg/kg i.p. on days 1 to 3), G-CSF (0.2 mg/kg s.c. on days 1 to 3), and/or AMD3100 (5 mg/kg i.p., day 4, 1 hour before blood collection). Blood was collected and analyzed for HSPCs by flow cytometry and CFU assay. IL-33 mobilized larger numbers of HSPCs than G-CSF or AMD3100, as indicated by increased numbers of LSK cells (A) and myeloid progenitor colony-forming units (B). Mean and range shown for each column (n = 2). IL-33 treatment significantly increased the number of mobilized HSPCs. Mann-Whitney test of IL-33-treated versus non-IL-33-treated animals (n = 8 per binned group indicated by brackets). $^*P < .05$. $^{***}P < .001$.

blood by a functional colony-forming assay for myeloid progenitor cells, we observed that IL-33 and G-CSF similarly mobilized myeloid progenitor cells consisting of early progenitor CFU-GEMM (for granulocytes, erythrocytes, monocytes, megakaryocytes) and more differentiated CFU-GM (for granulocytes, monocytes) and BFU-E (for erythrocytes), whereas AMD3100 alone did not appear to mobilize CFUs (Figure 1B). Even though AMD3100 alone did not show any benefit, combination of AMD3100 with either IL-33 or G-CSF resulted in a synergistic increase in mobilized LSK and myeloid progenitor cells (Figure 1). Interestingly, combination of IL-33 and G-CSF appeared additive, suggesting that IL-33 and G-CSF mobilize HSPCs via complementary mechanisms.

IL-33 Gets Inactivated by Oxidation

Full-length human IL-33 is composed of 2 domains: an *N*-terminal DNA-binding domain followed by an IL-1-like cytokine domain. The *N*-terminal domain can be cleaved off by proteases such as elastase, cathepsin G, and proteinase 3, releasing *N*-terminal-truncated mature forms that contain only the IL-1-like cytokine domain [20]. Compared with full-length IL-33, mature IL-33 forms are 10-fold more potent for ST2 activation [20]. Recombinant mature IL-33 (ie, the cytokine fraction of the molecule) is commercially available (sequence shown in Figure 2A). Highlighted on this sequence are the cysteine residues, which have been described to be subject to oxidation and subsequent inactivation of the molecule. These cysteine residues are also indicated on the structural model of IL-33 (Figure 2B). Comparing commercially available recombinant mature IL-33 proteins in a cell-based IL-33 reporter assay, we observed a wide range of activities (Supplementary Figure S1). Interestingly, 2 of the most active IL-33 proteins were purified in a buffer containing dithiothreitol, a reducing agent that can keep cysteines of IL-33 in their reduced form. Similarly, an oxidation-resistant IL-33 variant (IL-33[SCSC]), in which 2 of the 4 cysteines were mutated to serines, was also very active (3.3 ng/mL). We then confirmed by mass spectrometry that IL-33 containing 4 cysteines indeed was oxidized in nonreducing conditions, whereas the IL-33[SCSC] mutant was not (Supplementary Figure S2). To our surprise, a commercially available mammalian cell-expressed IL-33-Fc fusion protein was completely inactive (Supplementary Figure S1).

Oxidation-Resistant IL-33 Variants Mobilize HSPCs More Efficiently Than Nonmutated IL-33

Based on the oxidation sensitivity of the wild-type IL-33 cytokine, we designed an oxidation-resistant IL-33 cytokine variant (IL-33[SSSS]) in which all 4 cysteines are mutated to serines and expressed this variant, as well as nonmutated IL-33 in *Escherichia coli*. These 2 variants—IL-33[SSSS] (*E. coli*) and IL-33 (*E. coli*)—as well as all other variants generated throughout this study are listed for ease of reference in Supplementary Table S1. We next tested these 2 variants in a cell-based IL-33 reporter assay and observed that IL-33[SSSS] (*E. coli*) was approximately 14-fold more active than the nonmutated variant (Figure 3A, Supplementary Table S1).

Furthermore, we established a primary cell-based assay using HUVECs. It had been previously described that aortic fragments express CCL7 in response to IL-33 [17]. We hypothesized that the source of this chemokine may be endothelial cells within the aortic fragments. Previous studies suggested that HUVEC cells respond to stimulation with IL-33 [21], and we confirmed by flow cytometry that HUVEC cells express the IL1RL1 receptor chain (Figure 3B). When treating the cells with IL-33 variants and measuring CCL7 in supernatants, we observed that IL-33[SSSS] treatment resulted in increased CCL7 production compared with nonmutant IL-33 (Figure 3C).

Human and mouse IL-33 have approximately 55% amino acid sequence homology, and human IL-33 has been used successfully in mice [7]. In our own studies, human and mouse IL-33 mobilized HSPCs comparably (data not shown). We therefore selected C57BL/6 mice as our model system to compare IL-33 [SSSS] (*E. coli*) with nonmutated IL-33 (*E. coli*) and G-CSF in dose response studies in vivo. Animals were treated with indicated test articles at indicated dose levels once daily for 3 or 4 days (Figure 4). We observed that numbers of mobilized HSPCs (LSK),

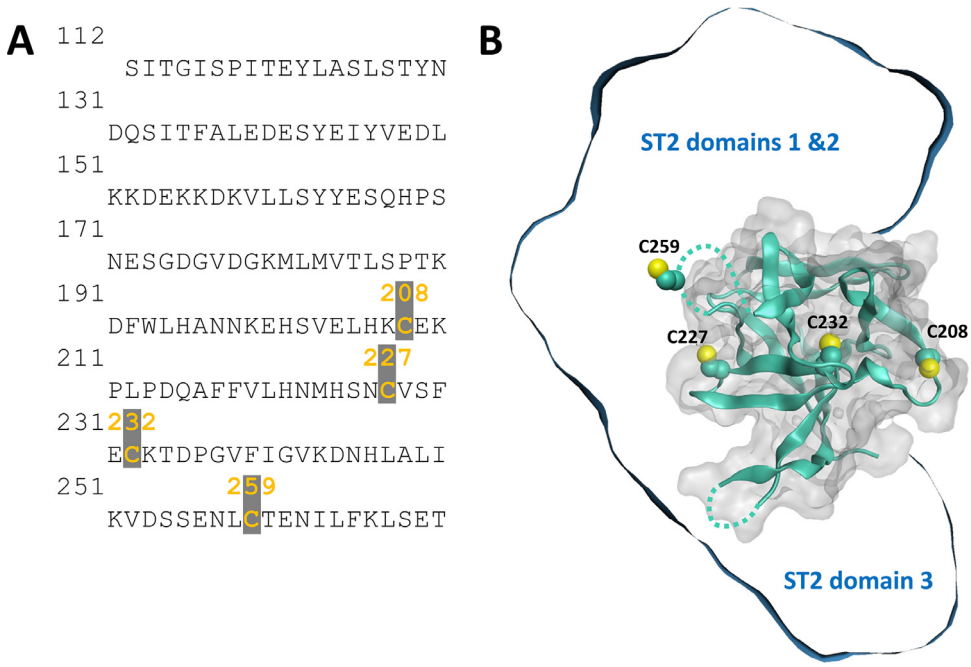

Figure 2. Human IL-33 has several cysteine residues subject to oxidation. The 4 cysteines are highlighted in the human IL-33 (A) sequence and (B) structure (protein data bank code: 4KC3). C259 is located at a disordered linker shown as a dotted line. Dark blue contour depicts ST2 extracellular domains when bound to IL-33.

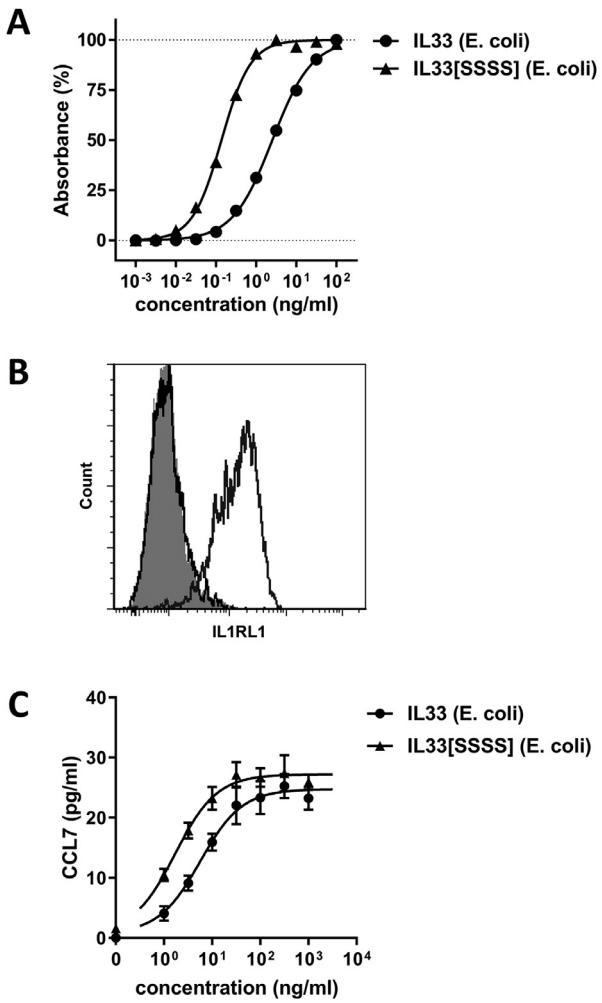

Figure 3. In vitro activity of *E. coli*-expressed IL-33 variants in cell-based IL-33 reporter and HUVEC assays. The *E. coli*-derived oxidation-resistant IL-33[SSSS] variant was more active than the wild-type molecule in a cell-based IL-33 reporter assay (A). HUVECs express the IL-33 receptor IL1RL1 (gray line) compared with isotype antibody-stained and unstained cells (black line overlaid with gray shading; B). Treatment of HUVECs with *E. coli*-derived IL-33 and IL-33[SSSS] dose-dependently increased the expression of CCL7 (C). Mean ± standard deviation. One representative experiment is shown (n = 2).

as well as long-term hematopoietic stem cells (HSCs; LSK $CD34^{-}$), were significantly increased in IL-33[SSSS]-treated animals compared with IL-33- or G-CSF-treated animals. This was observed after both 3 and 4 treatment days, whereas absolute numbers of mobilized cells were increased when animals were treated for 4 instead of 3 days. Mobilization of HSCs was observed at lower doses of IL-33[SSSS] than G-CSF. Importantly, we also observed significantly increased numbers of common lymphoid progenitors (CLPs) (LSK $CD127^{+}$) after IL-33[SSSS] treatment, suggesting that IL-33-mobilized HSPCs may result in a better lymphoid reconstitution compared with G-CSF. Interestingly, treatment with IL-33[SSSS] also resulted in mobilization of large numbers of endothelial progenitors, which may explain some of the benefits that have been described for IL-33 treatment in cardiovascular diseases.

IL-33 Variants Expressed as Secreted Proteins in Mammalian Cells Are Active In Vitro and In Vivo

Although mutating cysteines within IL-33 improved its activity, the IL-33 cytokine is an ~18-kDa molecule, much smaller than the kidney ultrafiltration cutoff of ~70 kDa, and can lead to fast clearance in vivo [22]. Improving pharmacokinetic properties of IL-33 may significantly reduce the number of injections required to mobilize HSPCs. To improve pharmacokinetics of smaller proteins, a few approaches have been used widely such as PEGylation [22], fusion to nonstructured polypeptides such as XTEN [23], or fusion to larger proteins such as either human serum albumin or the Fc domain of a human IgG [24]. We decided to create an IL-33-Fc fusion molecule, which may (a) increase the size and hydrodynamic radius of IL-33 to prevent rapid glomerular ultrafiltration and (b) allow pH-dependent FcRn-Fc binding to mediate recycling of the fusion protein back to circulation after pinocytosis, instead of undergoing lysosomal degradation. Both mechanisms may contribute to improvement of the pharmacokinetic half-life of the fusion molecule. Because proper folding of a functional Fc domain requires post-translational modifications such as intermolecular disulfide bond formation, we decided to express this fusion protein as a secreted protein in mammalian cells. To our knowledge, no active secreted IL-33 has been made in mammalian cells to date. In fact, the only commercially available CHO-derived IL-33 was inactive in our studies (Supplementary Figure S1).

As described above, IL-33 cytokine has 4 cysteines, which stay in their reduced form when the native IL-33 is expressed as a cytosolic protein in vivo. Upon oxidation, the cysteines form intramolecular disulfide bonds, and IL-33 becomes inactive. If we force IL-33 to enter the secretory pathway, as required for Fc folding, the molecule could form intra- and intermolecular disulfide bonds, which could lead to inactivation and oligomerization/aggregation.

We therefore expressed as a first step IL-33 and an IL-33 [SSCS] variant in mammalian cells, the latter of which has all 3 surface cysteines mutated to serines and thus conceptually may be constitutively active. When expressed as secreted protein, the N171 of wild-type IL-33 can potentially be *N*-link glycosylated according to sequence prediction. Based on modeling, N171 is located near the IL-33-IL1RAP interaction surface (Supplementary Figure S3), and thus its glycosylation may lead to lower activity of IL-33 because of steric hindrance. Besides, the IL-33[SSCS] variant introduces a potential second *N*-glycosylation site (N257) because of the C259S mutation. To characterize whether the wild-type and SSCS variants are glycosylated, we performed mass spectrometry analysis of the mammalian-expressed proteins and observed a complex glycosylation pattern (Supplementary Figure S4). Notably the glycosylation pattern was comparable between both wild-type IL-33 and the IL-33[SSCS] variant, indicating the second potential *N*-glycosylation site N257 in SSCS is not glycosylated, which was confirmed by peptide mapping (data not shown). This also suggests that any activity differences between the 2 molecules would solely be based on the cysteine-serine replacements. To test whether the glycosylation itself affected the activity of the proteins, we enzymatically deglycosylated the proteins and tested them in the cell-based IL-33 reporter assay (Figure 5A). As expected, IL-33[SSCS] was more active than nonmutated IL-33 by approximately 25-fold (Supplementary Table S1), whereas the deglycosylated protein was only slightly more active (Figure 5A), suggesting that the N171 glycosylation only minimally affected activity despite the glycan that may be close to IL1RAP. CCL7 secretion by primary HUVECs was also comparable for the glycosylated and enzymatically deglycosylated variants (Figure 5B).

We also tested the activity of these variants following overnight incubation at 37°C in the presence or absence of a

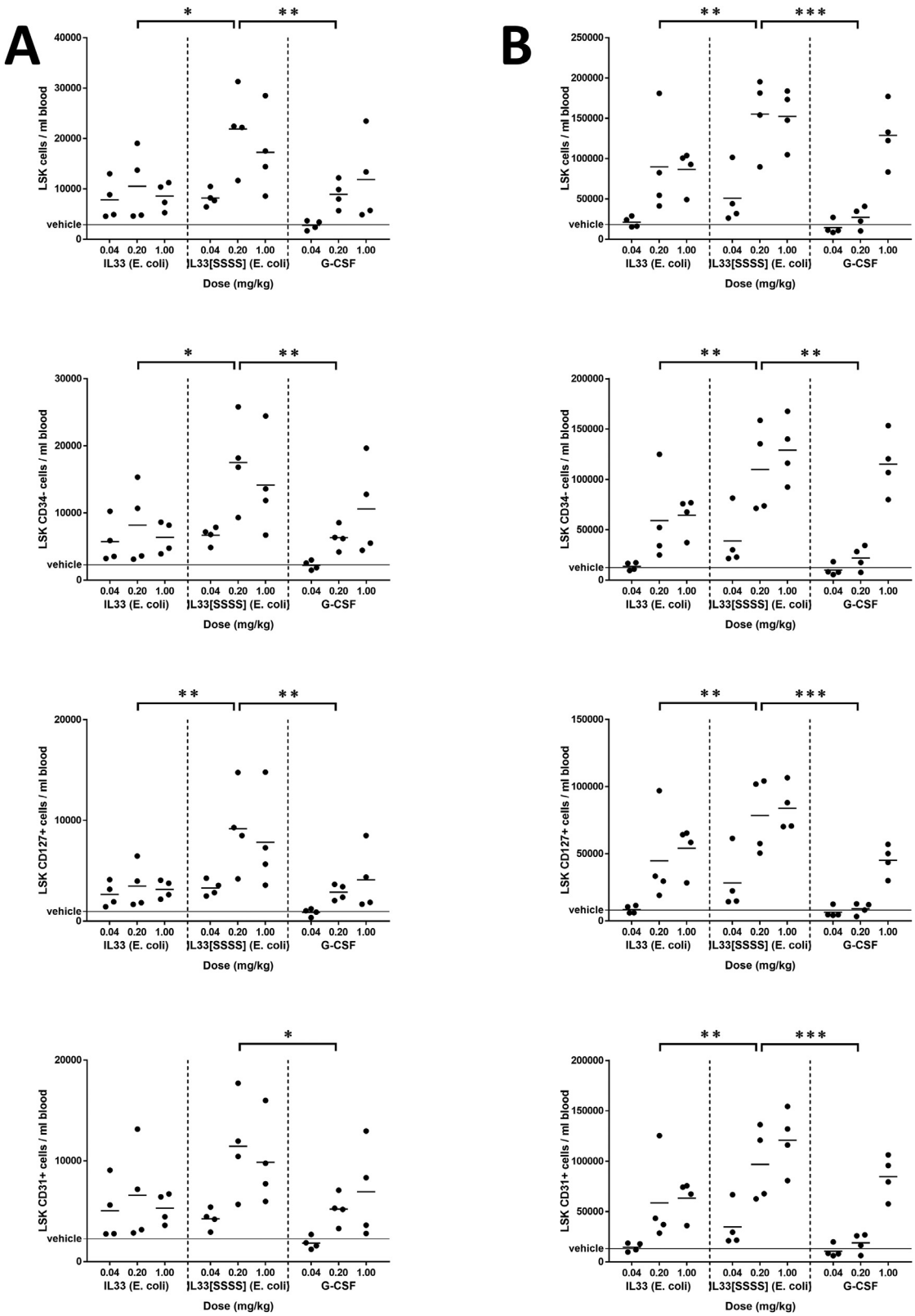

Figure 4. Oxidation-resistant IL-33 mobilizes stem and progenitor cells more efficiently than wild-type IL-33 or G-CSF. Mice were treated once daily for 3 days (A) or 4 days (B) with wild-type IL-33 (squares), oxidation-resistant IL-33[SSSS] (triangles), or G-CSF at the indicated doses. One day after the last dose, blood was collected and analyzed by flow cytometry. Significant increases in short- and long-term hematopoietic stem and progenitor cells (LSK; LSK CD34$^-$), common lymphoid progenitor cells (LSK CD127$^+$), and endothelial progenitor cells (LSK CD31$^+$) were observed. Data were analyzed by 2-way analysis of variance and Tukey post-test. $^*P < .05$. $^{**}P < .01$. $^{***}P < .001$.

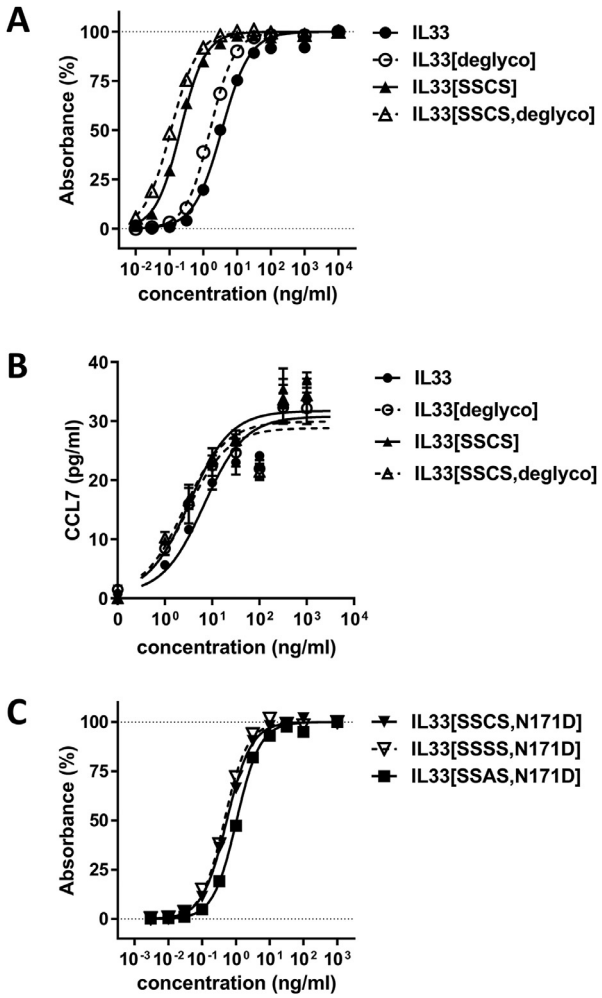

Figure 5. In vitro activity of mammalian-expressed IL-33 variants in cell-based IL-33 reporter and HUVEC assays. The oxidation-resistant IL-33[SSCS] variant was more active than the wild-type molecule when derived from a mammalian expression system (A). IL-33, IL-33[SSCS], and the respective deglyco variants similarly increased CCL7 secretion by HUVEC cells (B). Deglycosylation of the molecules with PNGase-F only minimally affected activity. N171D variants that lack an *N*-glycosylation site were active as well (C). Mean ± standard deviation. One representative experiment is shown (n = 2).

reducing agent. The nonmutated IL-33 control proteins lost several orders of magnitude activity in the absence of 2-mercaptoethanol, whereas the activity of the IL-33[SSCS] variants was only minimally affected (Supplementary Figure S5), providing further evidence that mutating surface cysteine residues may prevent inactivation of IL-33. The remaining cysteine residue, C232S, is buried inside of the IL-33 molecule based on the structure (Figure 2B) and most likely would not contribute to disulfide bond formation and subsequent inactivation of IL-33. To confirm this and further improve the activity of IL-33, we tested in parallel how mutating the remaining cysteine residue (C232) to either serine or alanine or removing the *N*-glycosylation site (N171G mutation) would affect activity of the variants. Although these changes did not appear to improve activity (Figure 5C), they negatively affected protein expression levels. Therefore, IL-33[SSCS] appears to be the best variant when considering both activity and expression level and was used as the base molecule for the subsequent studies. The protein could be enzymatically deglycosylated, which may further improve activity if needed. When treating C57BL/6 mice with IL-33[SSCS] and IL-33[SSCS,deglyco] once daily for 3 days in vivo, we observed an increase in mobilized HSCs, common lymphoid progenitors, and endothelial progenitors (Figure 6). The mobilization observed with the glycosylated IL-33[SSCS] was slightly smaller than with the deglycosylated protein, possibly because of slight activity differences that we had observed in the cell-based assays (Figure 5).

IL-33-Fc Mobilizes High-Quality HSPC Even after a Single Dose

We next engineered, expressed, and purified a 1-arm IL-33-Fc fusion molecule (Supplementary Table S1) comprising a single oxidation-resistant IL-33[SSCS] fused to a dimeric Fc domain using a knob-in-hole approach [25,26]. In brief, 2 peptide chains were coexpressed to create the 1-arm IL-33-Fc molecule, 1 chain for IL-33[SSCS]-Fc (knob) and the other chain for the Fc (hole)-only molecule. We chose this approach because a regular bivalent Fc fusion molecule with 2 IL 33 proteins per Fc molecule could theoretically crosslink the IL1RL1 receptor chain in absence of IL1RAP and thereby initiate a TIR-domain-mediated signaling cascade. Because IL1RL1 and IL1RAP may be differentially expressed, such a bivalent molecule may therefore activate cells differently from endogenous IL-33 and cause hard to predict cellular responses. In addition, as Fc effector function is not desired, we chose human IgG4 as the Fc backbone. We expressed and purified this 1-arm IL-33[SSCS]-Fc molecule with approximately 90% purity (Supplementary Figure S6). The activity of the 1-arm IL-33[SSCS]-Fc was approximately 2-fold equimolar range when correcting for the molecular weight difference between fusion and nonfusion proteins (Figure 7A). We further observed that the 1-arm IL-33-Fc fusion molecule caused CCL7 chemokine release in HUVEC cells similar to IL-33, whereas G-CSF did not (Figure 7B).

When dosing C57BL/6 mice with 1-arm IL-33[SSCS]-Fc once daily for 3 days, we observed a significant increase in mobilized HSCs, CLP, and endothelial progenitor cells, whereas dosing with IL-33[SSCS] at equal mass or equal molar amounts or dosing with G-CSF mobilized fewer cells (Figure 8A). Most important, we demonstrated that even a single dose of IL-33 [SSCS]-Fc, but not IL-33[SSCS], resulted in significant mobilization of HSCs, CLP, and endothelial progenitor cells (Figure 8B).

DISCUSSION

In this present study, we engineered recombinant IL-33 molecules that differ from endogenous IL-33 in several aspects. First, similar to commercially available IL-33 preparations that we tested (Supplementary Figure S1), we removed the *N*-terminal nuclear and activation domains. These domains may directly or indirectly regulate transcription and be involved in the enzymatic activation of IL-33 [27,28] but are not required for IL-33-mediated ST2 activation [20]. For our studies to develop an extracellular IL-33 therapeutic, we focused on the cytokine domain itself, which gets activated by enzymatic cleavage of the endogenous protein via various proteases in vivo to become the cytokine that activates the IL-33 receptor [20,29]. Additional IL-33 variants may be generated by alternative splicing [30,31].

We modified several cysteines within the IL-33 cytokine domain, which had been previously reported to be inactivation switches for IL-33 when oxidized [13]. By mutating the cysteines, the activity of IL-33 can be retained in the presence of oxidative stress, which may often occur during inflammation [32]. In this present study, we demonstrated that “oxidation-resistant” IL-33 molecules indeed retained their activity when exposed to ambient oxygen in vitro and had higher activity in vivo.

Having created an IL-33 molecule without an off-switch, we next wanted to express it as an Fc fusion to further extend its duration of action. Expression of the Fc domain required use of

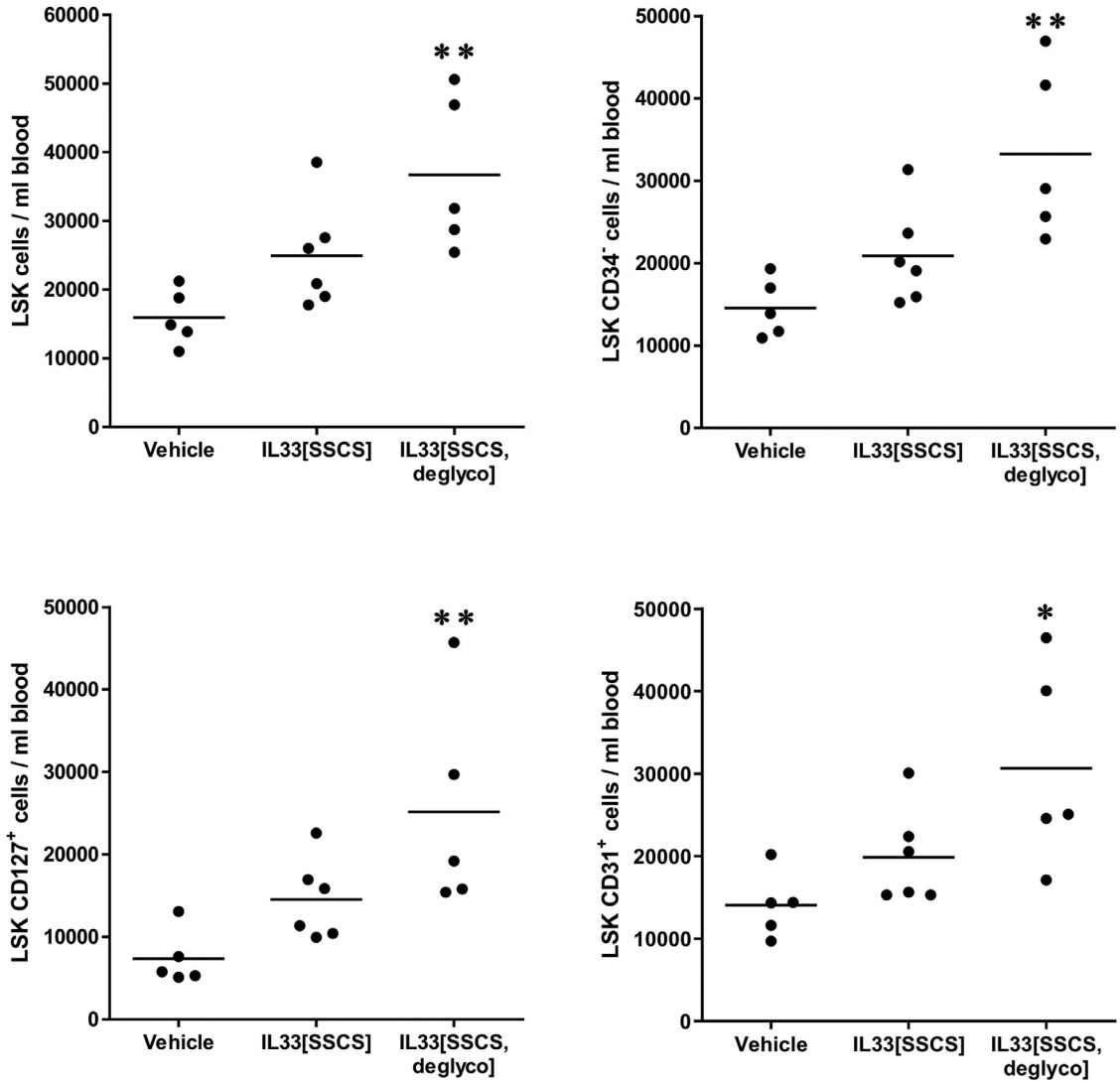

Figure 6. Mammalian-expressed IL-33 mobilizes stem and progenitor cells. C57BL/6 mice were dosed with IL-33[SSCS] or IL-33[SSCS,deglyco] (0.04 mg/kg) once daily for 3 days. Heparinized blood was collected at day 4 for flow cytometry analysis. Treatment with IL-33[SSCS,deglyco] significantly increased the mobilization of HSPCs (LSK and LSK CD34$^-$), CLP (LSK CD127$^+$), and endothelial progenitor cells (LSK CD31$^+$). Data were analyzed by analysis of variance and Dunnett's post-test. $^*P < .05$. $^{**}P < .01$.

a secreted mammalian expression system, whereas all previously reported active IL-33 molecules had been expressed in *E. coli*. Additionally, because IL-33 signals by heterodimer formation with IL1RL1 and IL1RAP, as well as subsequent phosphorylation of their intracellular TIR domains [33], a classical bivalent IL-33-Fc fusion molecule could possibly trigger TIR domain signaling by IL1RL1 crosslinking, which may cause signaling when IL1RL1 but not IL1RAP is expressed. Such a bivalent IL-33 molecule may therefore unspecifically trigger a signaling cascade in cells that naturally would not respond to IL-33 if these cells differentially express IL1RL1 in the absence of IL1RAP. Because we planned to mimic the function of IL-33, we decided to engineer a monovalent 1-arm IL-33-Fc fusion molecule comprising a single oxidation-resistant IL-33 fused to an Fc domain using a knob-in-hole approach [25]. The resulting monovalent molecule was within 2-fold activity compared with IL-33 and mobilized HSPCs even after a single dose, whereas equimolar amounts of IL-33 did not.

Using the molecules described, we tested the hypothesis whether IL-33 mobilizes HSPCs into the peripheral blood and compared it with clinical standard of care. The dose of G-CSF that we used for most of our studies is comparable to the dose typically used by others before [34–36]. The dose of 0.25 mg/kg/d has been described as a saturating dose to mobilize neutrophils when administered in 2 daily doses [37]. This previous study, however, did not provide a dose response for the mobilization of HSPCs, which we therefore performed as part of this present study. Although we observed that higher doses of G-CSF can indeed mobilize larger numbers of HSPCs, these numbers were nevertheless still smaller than the number of cells mobilized in response to IL-33.

Previous studies with IL-33 reported an increase in myeloid and B cell progenitors in peripheral organs, but these studies did not monitor HSCs directly [17]. In this present study, we demonstrated for the first time that IL-33 and its variants mobilize short- and long-term HSCs, as identified by LSK and LSK CD34$^-$ phenotypes, respectively [38]. Although this commonly used approach may be limited because mobilization agents may transiently change the CD34 phenotype of HSCs [39,40], it is supported by the previous report that IL-33 mobilized HSPCs, which improve long-term survival in bone marrow reconstitution studies, indicating that these cells contain a

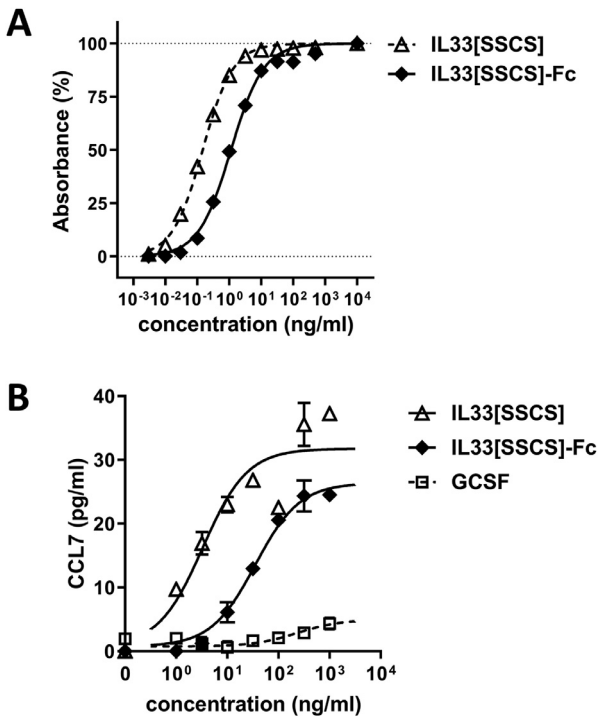

Figure 7. In vitro activity of mammalian-expressed IL-33-Fc fusion molecules in cell-based IL-33 reporter and HUVEC assays. IL-33[SSCS]-Fc-fusion protein is active within approximately 2 times equimolar range compared with non-Fc IL-33[SSCS] (A). IL-33[SSCS]-Fc and IL-33[SSCS] increased CCL7 expression, whereas G-CSF did not (B). Mean ± standard deviation. One representative experiment is shown (n = 2).

significant fraction of long-term HSCs [17]. Importantly, even though only about one-tenth of the LSK cells are actually stem cells [41], this condition is similar to human HSC transplants consisting of $CD34^+$ cells in which only about 1 in 200 cells has engraftment potential [42].

Early absolute lymphocyte count recovery, as a surrogate marker of immune recovery, is associated with prolonged survival [43]. CLP cells in mice have been identified by their LSK $CD127^+$ phenotype [44,45]. Using these surface markers, we demonstrated in this present study that IL-33 mobilizes CLP cells more efficiently than G-CSF. This correlates with previous reports that IL-33 treatment increased the number of CFUs for pre-B cells in peripheral organs [17] and that IL-33 promoted the egress of group 2 innate lymphoid cells from the bone marrow [46].

Furthermore, IL-33 has been linked to angiogenesis, increased proliferation, migration, and morphologic differentiation of endothelial cells [47]. This led us to hypothesize that IL-33 may also mobilize endothelial progenitor cells from the bone marrow. We therefore monitored in this present study LSK $CD31^+$ cells and observed that the numbers of these endothelial progenitor cells increased in mice treated with IL-33. IL-33 was considered protective in atherosclerosis and ischemic brain injury [15,48]. The contribution of endothelial progenitor cells to these diseases has been described extensively [49,50]. Future studies will have to evaluate the contribution of IL-33-mediated endothelial progenitor cell release to the cardiovascular benefits of IL-33 treatment.

Notably, the IL-33 variants that we used mobilized HSPCs not only more efficiently than G-CSF but also in an additive fashion, suggesting IL-33 mobilizes HSPCs through a mechanism distinct from G-CSF. The highly complex mechanism by which G-CSF mobilizes HSPCs is indirect and in part by reducing CXCL12 expression and alteration of the HSC niche, which permits HSCs to leave into the peripheral circulation [51]. In contrast, we and others [17] demonstrated that IL-33 may mediate HSC mobilization at least in part through another chemokine, CCL7. These different mechanisms may explain the additive effect between G-CSF and IL-33. Although anti-CCL7 antibodies nearly completely blocked the IL-33-mediated HSPC mobilization [17], previous work treating umbilical cord-derived $CD34^+$ cells with IL-33 resulted in increased T helper 2 cytokine production, suggesting a possible direct effect of IL-33 on HSPCs [52], which remains to be explored in future studies.

Recent reports further indicated that IL1RL1 may be dynamically expressed in certain leukemia stem cells and that IL-33 treatment may promote leukemia stem cell survival [53]. If IL1RL1 is predominantly expressed in leukemia stem cells, IL-33 could be linked to clonal hematopoiesis of indeterminate potential (CHIP) in older donors [54]. The risk of donor-derived myelodysplastic syndrome has been described and correlated with CHIP in a study using G-CSF-mobilized HSPCs [55]. A possible link between IL-33 and the most common CHIP mutations (*DNMT3A*, *TET2*, and *ASXL1* genes) has not been described to date and could be an interesting topic for future studies.

When using AMD3100 with IL-33 or G-CSF, we observed a pronounced synergistic effect even when AMD3100 was given just 1 hour before blood collection. This fairly quick effect has been previously described [56,57] and may explained by AMD3100 not only directly affecting the bone marrow to release HSPCs but also by AMD3100 hindering the homing of these cells to secondary lymphoid organs, particularly the spleen, thereby synergistically increasing the number of circulating HSPCs in the blood when combined with other agents [51]. Other mobilization agents that are in preclinical and clinical development target the bone marrow niche either directly or indirectly via the CXCR4-CXCL12 axis like AMD3100, via other chemokine receptors or retention factors, or by addition of growth factors, cytokines, or hormones [58,59]. To our knowledge, none of these investigational factors have been reported to have an encouraging mobilizing profile like IL-33.

In summary, IL-33 mobilizes HSPCs more efficiently than G-CSF or AMD3100 alone and is additive or synergistic in combination with the other therapies. Oxidation-resistant IL-33 variants have greater activity in vitro and in vivo than wild-type IL-33 and can be engineered as a 1-arm IL-33-Fc fusion molecule that is long-acting. IL-33 triggers secretion of CCL7 from endothelial cells, which likely contributes to the mobilization of HPSCs that contain HSCs, CLP, and endothelial progenitor cells.

In conclusion, long-acting, oxidation-resistant IL-33 may be a novel approach for HSPC transplantation. IL-33-mobilized HSPCs differ from cells mobilized with G-CSF and AMD3100, and it is possible that these differences may result in better transplantation outcomes, which remain to be demonstrated in future studies.

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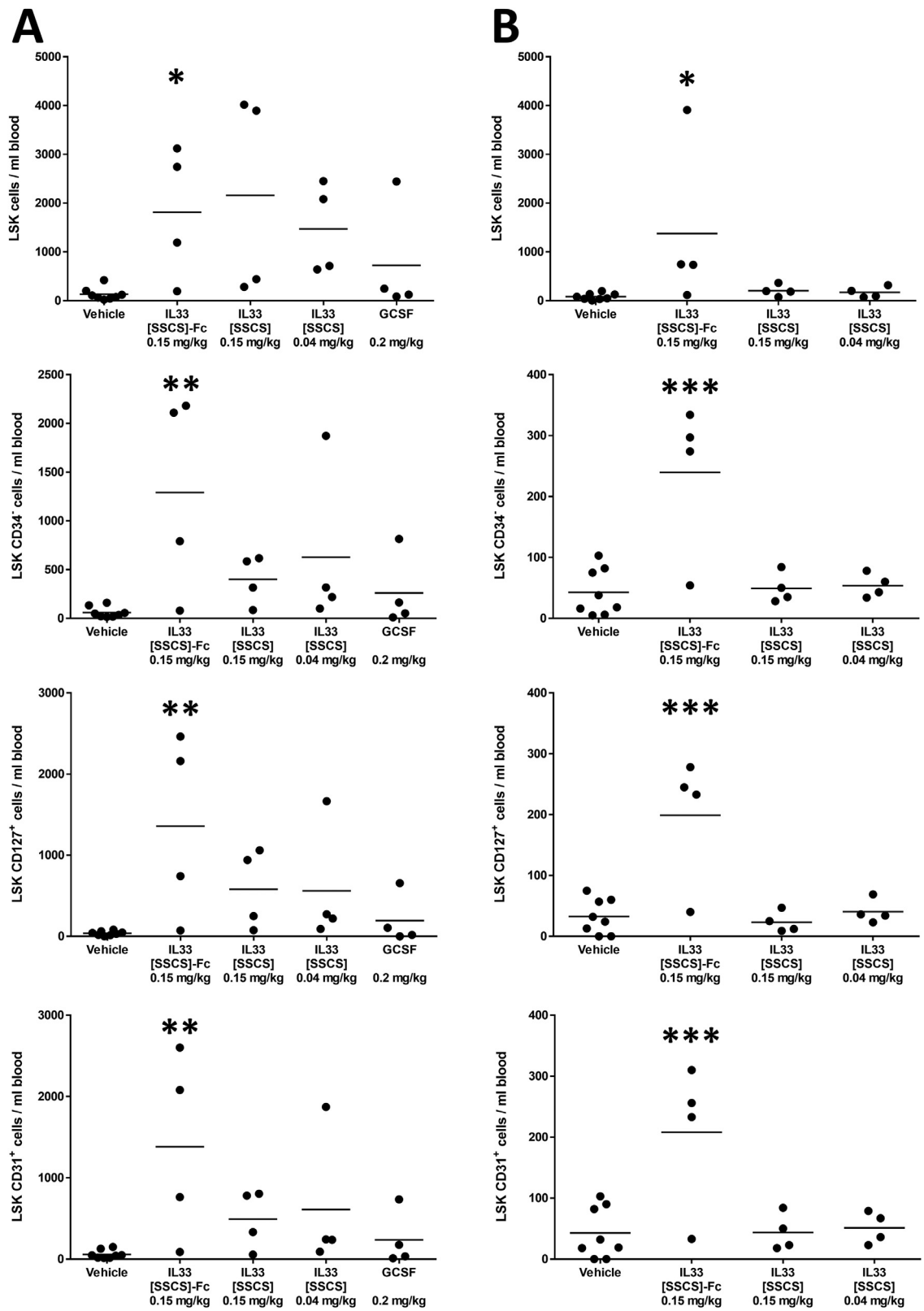

Figure 8. Long-acting IL-33[SSCS]-Fc mobilizes LSK, CLP, and endothelial progenitor cells. C57BL/6 mice were dosed with IL-33[SSCS]-Fc (0.16 mg/kg i.p.) or IL-33 [SSCS] at equal mass (0.16 mg/kg i.p.) or approximately equal molar amount (0.04 mg/kg i.p.). G-CSF treatment at previously efficacious dose (0.2 mg/kg s.c.) was used as a control. Animals were treated once daily for 3 days (A) or once on day 1 (B). Heparinized blood was collected at day 4 for flow cytometry analysis. Treatment with IL-33[SSCS]-Fc significantly increased the mobilization of HSPCs (LSK and LSK CD34$^-$), CLP (LSK CD127$^+$), and endothelial progenitor cells (LSK CD31$^+$), even after a single treatment. Data were analyzed by analysis of variance and Dunnett's post-test. $^*P < .05$. $^{**}P < .01$. $^{***}P < .001$.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2019.05.030.

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