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## Nov/CCN3 regulates long-term repopulating activity of murine hematopoietic stem cells via integrin $\alpha v \beta 3$

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**Abstract**

Throughout life, hematopoietic stem cells (HSCs) sustain the blood cell supply through their capacities for self-renewal and multilineage differentiation. These processes are regulated within a specialized microenvironment termed the 'niche'. Here, we show a novel mechanism for regulating HSC function that is mediated by nephroblastoma overexpressed (*Nov/CCN3*), a matricellular protein member of the CCN family. We found that *Nov* contributes to the maintenance of long-term repopulating (LTR) activity through association with integrin  $\alpha\beta3$  on HSCs. The resultant  $\beta3$  integrin outside-in signaling is dependent on thrombopoietin (TPO), a crucial cytokine involved in HSC maintenance. TPO was required for *Nov* binding to integrin  $\alpha\beta3$ , and stimulated *Nov* expression in HSCs. However, in the presence of  $\text{IFN}\gamma$ , a cytokine known to impair HSC function, not only was TPO-induced expression of *Nov* suppressed, but the LTR activity was conversely impaired by TPO-mediated ligation of integrin  $\alpha\beta3$  with exogenous ligands, including *Nov*, as well. Thus, *Nov*/integrin  $\alpha\beta3$ -mediated maintenance of HSCs appears to be modulated by simultaneous stimulation by other cytokines. Our finding suggests that this system contributes to the regulation of HSCs within the bone marrow niche.

**Keywords**

Hematopoietic stem cell; Nephroblastoma overexpressed; Integrin  $\alpha\beta3$ ; Thrombopoietin

**Introduction**

Hematopoietic stem cells (HSCs) are responsible for the life-long maintenance of the hematopoietic system both by sustaining HSCs through self-renewal and by giving rise to the various blood cell lineages via stepwise differentiation. To maintain this capacity, HSCs reside in a specialized microenvironment within the bone marrow (BM) termed a 'niche,' where their functions are appropriately regulated via complex combinations of mechanisms, including cytokine signaling, signaling via cell-cell contact, and cell-extracellular matrix (ECM) adhesion [1].

We recently reported a novel mechanism of integrin  $\alpha\beta3$  outside-in signaling that is indispensable for the regulation of the long-term repopulating (LTR) activity of HSCs [2]. This integrin function was dependent on the presence of thrombopoietin (TPO), an essential cytokine that contributes to HSC maintenance [3–5] and promotes  $\alpha\beta3$  integrin activation (a conformational change increasing its ligand affinity) through induction of integrin inside-out signaling (a pathway that activates integrins in response to extracellular stimulation) [2]. These findings established a mechanistic link between integrin  $\alpha\beta3$  signaling and TPO in maintaining the LTR activity of HSCs.

Nephroblastoma overexpressed (Nov/CCN3) is a soluble factor that belongs to the CCN family and is reportedly involved in modulating several biological processes including cell proliferation, cell adhesion, wound healing, and angiogenesis, through association with integrins, including integrin  $\alpha\text{v}\beta\text{3}$  [6–8]. Recently, human HSCs were reported to express higher *Nov* levels than other progenitor cells, suggesting that *Nov* expression may be a hallmark of HSCs [9, 10]. In addition, endogenous *Nov* expression in HSCs seems to be required for the maintenance of HSC function *in vivo* because the knockdown of *Nov* expression impaired the repopulating activity of human HSCs [9]. Furthermore, the addition of recombinant *Nov* was shown to enhance the repopulating activity of human HSCs cultured *ex vivo* [9]. These data suggest that *Nov* acts as a positive regulator of HSC function, however, the underlying mechanism by which *Nov* regulates HSC function remains unclear.

Interferon- $\gamma$  (IFN $\gamma$ ) is a cytokine that modulates immune systems and inflammation. While IFN $\gamma$  reportedly promotes HSC proliferation *in vivo* by prompting dormant HSCs to enter the cell cycle, this response is accompanied by an impaired maintenance of LTR activity [11]. The negative regulation of LTR activity by IFN $\gamma$  is mediated through STAT1 activation. Another recent report showed that IFN $\gamma$  directly impairs the proliferative capacity of HSCs *in vitro*, thereby suppressing LTR activity [12]. It thus appears that IFN $\gamma$  contributes to the maintenance of hematopoietic homeostasis through negative regulation of HSC function.

In this study, we identify a novel mechanism through which *Nov* regulates murine HSC function. *Nov* functions as a ligand of integrin  $\alpha\text{v}\beta\text{3}$  on HSCs, thereby contributing to the maintenance of LTR activity, which is dependent on TPO. In the presence of IFN $\gamma$  however, the ligation of integrin  $\alpha\text{v}\beta\text{3}$  with *Nov* or ECM exerts negative influences on HSC function. These findings demonstrate for the first time that *Nov* regulates HSC function via integrin  $\alpha\text{v}\beta\text{3}$  in the presence of simultaneous stimulation by other cytokines.

## Materials and methods

### Animals

The animals used in this study are described in “Supplemental Methods”.

### Antibodies

The antibodies used in this study are described in “Supplemental Methods”.

### Cell sorting and flow cytometric analyses

A MoFlo XDP or Gallios flow cytometer (Beckman Coulter Inc., Brea, CA) was used for cell sorting and flow cytometric analyses as described previously [2].

### Estimation of *Nov* binding on HSCs

CD34<sup>+</sup>KSL HSCs were cultured in S-Clone SF-03 medium (Eidia Co., Ltd., Tokyo, Japan) supplemented with 0.5 % bovine serum albumin (Sigma-Aldrich Corporation, St. Louis, MO), 1 mM MnCl<sub>2</sub> (Wako Junyaku, Osaka, Japan), 50 ng/ml TPO (R&D Systems,

Minneapolis, MN), and/or 5 ng/ml IFN $\gamma$  (Shenandoah Biotechnology Inc., Warwick, PA) for either 1 or 18 h prior to treatment with 2  $\mu$ g/ml recombinant mouse (rm)Nov tagged with oligohistidine (His-10) (R&D Systems) for 1 h. To identify bound rmNov, cultured cells were stained with an Alexa Fluor 647-conjugated mouse antibody recognizing the His-tag (AbD Serotec, Kidlington, UK). Subsequently, the stained cells were subjected to flow cytometric analyses after they were washed twice with PBS. For the inhibitory experiments, 200  $\mu$ M of an Arg-Gly-Asp-Ser (RGDS) synthetic peptide (Life Technologies, Inc., Carlsbad, CA) or 50  $\mu$ g/ml of antibodies against the integrins  $\beta$ 3 or  $\alpha$ v were added to the culture media, 1 h prior to the addition of rmNov. The Arg-Gly-Glu-Ser (RGES) peptide (Life Technologies, Inc.), Hamster IgG, or Rat IgG served as controls. All antibodies were purchased from BioLegend (San Diego, CA) unless otherwise indicated.

### HSC cultures

Sorted CD150<sup>+</sup>CD34<sup>-</sup>KSL HSCs were cultured for 5 days in S-Clone SF-03 serum-free medium supplemented with 50 ng/ml rmNov, 50 ng/ml mouse TPO, 50 ng/ml mouse SCF (R&D Systems), and/or 5 ng/ml mouse IFN $\gamma$ . Vitronectin (VN)-coated plates were prepared as described previously [2]. Following ex vivo culture, the total cell numbers were quantified by phase contrast microscopy, and subjected to the following experiments.

### Long-term competitive repopulation assays

Long-term competitive repopulation assays were performed as described previously [13]. Briefly, cultured HSCs together with  $2 \times 10^5$  whole BM competitor cells were transplanted into lethally irradiated (10 Gy) C57BL/6-Ly5.2 or C57BL/6-Ly5.1 congenic mice. Twenty weeks after transplantation, donor cell chimerism in the recipient mice was analyzed by flow cytometry. Recipient mice with donor cell chimerism (>1.0 % for myeloid and B- and T-lymphoid lineages) were considered to be multilineage-reconstituted mice.

### Estimation of the number of retained HSCs after culture (limiting dilution assay)

Samples containing 20, 40, 80, or 150 CD150<sup>+</sup>CD34<sup>-</sup>KSL HSCs were sorted into individual wells of a 96-well plate and cultured for 5 days under the indicated conditions. Subsequently, whole cultured cells from each well were used for the transplantation assays as described above. Twenty weeks after transplantation, the percentage of unreconstructed mice (negative mice) was determined and the number of primary CD150<sup>+</sup>CD34<sup>-</sup>KSL cells required to retain one cell with LTR activity after culture was estimated based on the Poisson distribution [14].

### Quantitative real-time RT-PCR

Using 5,000 sorted cells from each sample, mRNA expression was analyzed by quantitative real-time RT-PCR as previously described [2, 13]. This procedure is described in detail in the “Supplemental Methods”.

### Analyses of STAT activation

After cytokine stimulation, the STAT5 or STAT1 activation levels were determined by flow cytometric analyses. This procedure is described in detail in the “Supplemental Methods”.

## Statistical analysis

The results are expressed as mean  $\pm$  standard deviation (SD). Statistical significance between two groups was assessed by the unpaired Student's *t* test. *P* values  $<0.05$  were considered significant.

## Results

### Nov contributes to the maintenance of the TPO-dependent LTR activity of HSCs through integrin $\alpha v\beta 3$

We previously demonstrated that outside-in signaling via integrin  $\alpha v\beta 3$  plays a key role in the maintenance of HSC LTR activity [2]. Because Nov is a known integrin  $\alpha v\beta 3$  ligand [6], we hypothesized that Nov acts via integrin  $\alpha v\beta 3$  to regulate LTR activity. To address this possibility, we first confirmed that Nov associates with integrin  $\alpha v\beta 3$  using flow cytometric analyses of CD34<sup>-</sup>KSL HSCs treated with His-tagged rmNov. By staining with an antibody recognizing the His-tag, enhanced binding of rmNov could be detected when HSCs were simultaneously treated with rmNov and Mn<sup>2+</sup>, a known integrin activator [15], whereas little binding of rmNov to HSCs was observed in the absence of Mn<sup>2+</sup> (Fig. 1a). In addition, the effect of Mn<sup>2+</sup> was inhibited by the Arg-Gly-Asp-Ser (RGDS) peptide, an integrin  $\alpha v\beta 3$  recognition sequence, but not by the Arg-Gly-Glu-Ser (RGES) control peptide. Furthermore, an anti-integrin  $\beta 3$  blocking antibody [16, 17] recapitulated the inhibitory effect of the RGDS peptide on Mn<sup>2+</sup>-dependent binding of rmNov to HSCs. These results indicate that Nov is capable of associating with integrin  $\alpha v\beta 3$  on HSCs, which is dependent on the activation of this integrin. We previously demonstrated that TPO contributes to the activation of integrin  $\alpha v\beta 3$  on HSCs through inside-out signaling [2], suggesting that TPO may mediate the association between Nov and integrin  $\alpha v\beta 3$  on HSCs. To confirm this hypothesis, we examined whether rmNov binds to cultured HSCs in the absence or presence of TPO. Although treatment with TPO for 1 h had little influence on rmNov binding to HSCs (Fig. 1b), extending the treatment with this cytokine to 18 h significantly enhanced rmNov ligation (Fig. 1c). In addition, the effect of TPO was inhibited in the presence of blocking antibodies against either the  $\beta 3$  or  $\alpha v$  integrin subunits (Fig. 1c) [16, 18]. These results indicate that TPO enables the association between Nov and integrin  $\alpha v\beta 3$  on HSCs and suggest that TPO and/or outside-in signaling via the integrin  $\alpha v\beta 3$  are involved in the Nov-mediated regulation of LTR activity.

Initially, to confirm whether TPO is involved in regulation of HSC functions by Nov, we performed transplantation assays using HSCs incubated with or without rmNov in the presence of TPO or stem cell factor (SCF), an essential cytokine for HSC maintenance that acts independently of integrin  $\alpha v\beta 3$  [2] (Fig. 2a). Twenty weeks after transplantation, HSCs cultured with rmNov in the presence of TPO showed greater chimerism than cells cultured in the presence of TPO alone (Fig. 2a). In contrast, rmNov had no effect on the LTR activity of HSCs cultured in the presence of SCF (Fig. 2a). These results indicate that the positive effect of Nov on LTR activity is dependent on the presence of TPO. In addition, after the culture with TPO, rmNov had no effect on the total cell number, or on the frequency of KSL and CD48<sup>-</sup>KSL cells, which are the HSC/hematopoietic progenitor cell (HPC) fraction, or the HSC-enriched populations, respectively (Supplemental Fig. 1) [19]. Thus, Nov is not

involved in HSC expansion, at least during ex vivo culture. Collectively, these results suggest that *Nov* positively regulates the LTR activity of individual HSCs, but it has no effect on HSC expansion. This behavior is reminiscent of the behavior of other integrin  $\alpha\beta3$  ligands [2] and strongly supports the hypothesis that *Nov* acts via integrin  $\alpha\beta3$  to regulate HSC function.

We have previously shown that outside-in signaling is indispensable for the sustained LTR activity mediated by  $\beta3$  integrin [2]. Therefore, to directly confirm that outside-in signaling via integrin  $\alpha\beta3$  is crucial for the positive effect of *Nov* on LTR activity, we carried out transplantation assays using HSCs derived from  $\beta3$  integrin Y747A knock-in mutant mice. In these mice, an alanine was substituted for tyrosine 747 in the  $\beta3$  integrin subunit, thereby eliminating outside-in signaling [20–22]. We found that the positive effect of *rmNov* on LTR activity was almost completely lost in HSCs expressing the Y747A-mutated  $\beta3$  integrin, even in the presence of TPO (Fig. 2b). These data suggest that, such as TPO, outside-in signaling via integrin  $\alpha\beta3$  is indispensable for the *Nov*-mediated regulation of LTR activity.

### **TPO contributes to the maintenance of expression of *Nov* in HSCs**

We have thus demonstrated the linkage between *Nov*, integrin  $\alpha\beta3$ , and TPO in HSCs. The expression of *Nov* is regarded as a hallmark of HSCs [9, 10] and is prompted by STAT5 activation [23]. Because TPO is capable of inducing STAT5 activation in HSCs [24], we suggest a further linkage between TPO and *Nov* in regard to the regulation of *Nov* expression in HSCs. To test this hypothesis, we first used quantitative real-time RT-PCR to determine whether TPO stimulates the expression of *Nov* in cultured HSCs. We found that HSCs cultured for 24 h in the presence of TPO exhibited sustained levels of *Nov* expression compared to freshly isolated HSCs (Fig. 3a). TPO treatment was also associated with STAT5 phosphorylation (Fig. 3b). Furthermore, *Lnk*<sup>-/-</sup> HSCs, which display prolonged activation of STAT5 in response to TPO treatment (Fig. 3c) [25], exhibited enhanced *Nov* expression compared to Wt HSCs not only after treatment with TPO but also in vivo (Fig. 3d). In contrast, HSCs cultured in the presence of SCF or under cytokine-free conditions do not express *Nov* and exhibit minimal STAT5 activation (Fig. 3a, b). These data suggest that TPO enables the induction of *Nov* expression in HSCs likely through STAT5 activation, as predicted from the previous reports [23, 24]. Interestingly, although neither freshly isolated Wt nor *Lnk*<sup>-/-</sup> CD34<sup>+</sup>KSL HPCs expressed *Nov*, TPO treatment of HPCs in vitro induced *Nov* expression and STAT5 activation. These effects were more pronounced in *Lnk*-deficient HPCs (Fig. 4). TPO thus appears to have the capacity to induce *Nov* expression in both HSCs and HPCs, suggesting that expression of *Nov* is dependent on external stimulation rather than the cell-autonomous phenotypes.

### **IFN $\gamma$ impairs the TPO-induced expression of *Nov* in HSCs through the activation of STAT1**

In response to cytokine stimulation, activated STAT5 is reportedly recruited into IFN $\gamma$ -activated sequence (GAS) sites within the promoter region of *Nov*, and that this event correlates with *Nov* expression [23]. Because GAS sites were originally identified as STAT5 or STAT1 binding motifs that modulate IFN $\gamma$ -activated transcription [26], we suspected that STAT1 was also involved in the regulation of STAT5-dependent *Nov* expression in HSCs.

To test this possibility, we examined *Nov* expression in HSCs treated simultaneously with TPO and IFN $\gamma$ , a known inducer of STAT1 activation. We found that IFN $\gamma$  treatment impaired TPO-dependent *Nov* expression in both HSCs (Fig. 5a) and HPCs (Data not shown). Moreover, the suppressive effect of IFN $\gamma$  was abrogated by treatment with epigallocatechin gallate (EGCG), an inhibitor of STAT1 activation (Fig. 5) [27]. Thus, the negative effect of IFN $\gamma$  on *Nov* expression in HSCs appears to be dependent on STAT1 activation, supporting the possibility that *Nov* expression is dependent on exogenous stimulation in both HSCs and HPCs.

### **Nov inhibits LTR activity via integrin $\alpha\beta3$ in the presence of IFN $\gamma$**

IFN $\gamma$ -mediated STAT1 activation reportedly leads to the suppression of HSC LTR activity as well as to an impaired HSC proliferation capacity in vitro [12]. Likewise, we confirmed that IFN $\gamma$  negatively affects both the proliferation capacity (Fig. 6a) and the LTR activity of HSCs cultured in the presence of TPO (Fig. 6b). Our findings indicate that IFN $\gamma$  dominantly impairs TPO-dependent expression of *Nov* (Fig. 5a), a positive regulator of LTR activity that may also be involved in the IFN $\gamma$ -mediated suppression of the repopulating activity of HSCs (Fig. 6b) [9]. We therefore reasoned that the addition of rmNov to HSCs cultured in the presence of TPO likely prevented the IFN $\gamma$ -dependent impairment of LTR activity by both offsetting the IFN $\gamma$ -induced reduction in *Nov* expression and by acting as a positive regulator of LTR activity. To test this possibility, we performed transplantation assays using HSCs cultured with or without rmNov in the presence of both TPO and IFN $\gamma$ . Surprisingly, the addition of rmNov appeared to exert negative effects on both the proliferation capacity and the maintenance of HSCs, as evidenced by a decreased total cell number, suppressed chimerism and an increase in the percentage of unreconstructed mice (Fig. 6a, b). To quantify the negative effects on HSC maintenance, we next performed a limiting dilution assay based on the transplantation assays. Consequently, this assay showed that approximately threefold the number of primary CD150<sup>+</sup>CD34<sup>-</sup>KSL cells is required to retain one cell with LTR activity when cells are cultured in the presence of IFN $\gamma$  and treated with rmNov (Fig. 6c). The number of HSCs decreased to approximately one-third compared with the control when cultured in the presence of IFN $\gamma$  and treated with rmNov (Fig. 6d). Therefore, the suppressive actions of rmNov on cell proliferation (Fig. 6a) are less likely to contribute to its negative effects on HSC maintenance because they are insufficient to account for the decrease in the estimated HSC number after culture. Thus, these results indicate that *Nov* negatively affects the maintenance of LTR activity in the presence of IFN $\gamma$ , even when TPO is present. In addition, we confirmed that IFN $\gamma$  had little effect on the TPO-dependent association of rmNov with  $\beta3$  integrin (Supplemental Fig. 2), suggesting that integrin  $\alpha\beta3$  is also involved in the negative actions of *Nov* observed in the presence of IFN $\gamma$ . To confirm this hypothesis, we examined the IFN $\gamma$ -dependent effects of rmNov in HSCs derived from integrin  $\beta3$  mice. The addition of rmNov to integrin  $\beta3$  HSCs cultured in the presence of both TPO and IFN $\gamma$  had little effect on HSC proliferation (Fig. 6e). Similarly, integrin  $\beta3$  deficiency abolished the IFN $\gamma$ -dependent negative effects of rmNov on LTR activity that were observed in Wt HSCs (Fig. 6f). Thus, such as its positive effects on HSC function in the presence of TPO, the negative effects of *Nov* in the presence of IFN $\gamma$  also appear to be mediated through integrin  $\alpha\beta3$ .

Finally, we confirmed whether another integrin  $\alpha\beta3$  ligand had similar negative effects on HSC function in the presence of IFN $\gamma$  using HSCs cultured on plates coated with vitronectin (VN), a well-known integrin  $\alpha\beta3$  ligand. Although VN coating had little influence on the proliferative capacity of HSCs in the presence of IFN $\gamma$  (Fig. 7a), transplantation assays demonstrated that this ligand recapitulates the negative effects of rmNov on the maintenance of LTR activity (Fig. 7b – d). These results strongly suggest a novel function of integrin  $\alpha\beta3$  that negatively affects HSC maintenance in the presence of IFN $\gamma$ .

## Discussion

In this study, we demonstrate that Nov acts via integrin  $\alpha\beta3$  on HSCs to bidirectionally regulate LTR activity in vitro, which is dependent on TPO-dependent activation of this integrin as well as simultaneous stimulation by other cytokines (Fig. 8). The observed positive effects of Nov on the maintenance of HSC LTR activity in the presence of TPO (Fig. 2a) are consistent with earlier findings [2, 9], while our observation that Nov negatively affects LTR activity in the presence of IFN $\gamma$  (Fig. 6) is novel. The negative behavior of Nov in the presence of IFN $\gamma$  likely reflects a novel mechanism by which integrin  $\alpha\beta3$  regulates HSC function. Consistent with this possibility, VN, a well-known integrin  $\alpha\beta3$  ligand, also recapitulates the behavior of Nov in the presence of IFN $\gamma$  (Fig. 7). Our findings thus provide a new understanding of the relationship between integrin and cytokine signaling in the regulation of HSC function.

Previously, *Nov* was thought to be exclusively expressed by HSCs in vivo and was thus considered a hallmark of those cells (Figs. 3a, 4) [9, 10]. However, our findings indicate that *Nov* expression does not seem to be an attribution of HSCs because *Nov* expression was evident in TPO-treated HPCs in vitro (Fig. 4) and SCF-treated cells lacking *Nov* expression were capable of engraftment (Figs. 2a, 3a). In addition, our results indicate that *Nov* expression in HSCs and HPCs is strongly influenced by cytokine stimulation (Figs. 3, 4, 5). These results suggest that the distribution of *Nov* expression in vivo is attributable to the environment surrounding each cell. Accordingly, we propose that TPO mediates *Nov* expression by HSCs in vivo because HSCs reportedly make contact with TPO-expressing cells within the osteoblastic niche [28]. Our data demonstrate that TPO supports both STAT5 activation and *Nov* expression in HSCs (Fig. 3). Moreover, *Nov* expression in vivo was increased by Lnk deficiency, which causes prolonged TPO-dependent STAT5 activation (Fig. 3c, d). These findings strongly suggest that TPO contributes to *Nov* expression in vivo. This possibility is also supported by a previous study showing that the positive effects of Lnk deficiency on the activation of JAK2, a signaling molecule that acts upstream of STAT5, are dependent on TPO stimulation but are unaffected by IL-3 or G-CSF [29].

Although other cells that likely compose the HSC niche, such as osteoblasts and vascular endothelial cells, also express *Nov* [30, 31], endogenous *Nov* expression in HSCs seems to have a stronger influence on the maintenance of HSC function within the BM niche because the knockdown of *Nov* expression in human HSCs leads to a decreased repopulating activity in vivo [9]. Indeed, this finding indicates that *Nov* secreted by the HSCs contributes to the maintenance of stem cell activity in vivo. Our findings demonstrate that TPO induces *Nov*



expression in vitro (Fig. 3), suggesting that TPO may regulate *Nov* expression in vivo. In addition, the positive effects of *Nov* on LTR activity in the presence of TPO seem to be mainly attributable to  $\beta 3$  integrin outside-in signaling (Fig. 2b). Taken together, our findings suggest that endogenous *Nov*, along with external ligands, likely acts via  $\alpha v\beta 3$  integrin on HSCs to contribute to the maintenance of LTR activity within a TPO-enriched niche.

In the presence of  $IFN\gamma$ , a cytokine that impairs HSC function, *rmNov* and *VN* exerted suppressive effects on HSC function in vitro, even in the presence of TPO (Figs. 6, 7). These results imply that integrin  $\alpha v\beta 3$  ligands preferentially contribute to  $IFN\gamma$ -dependent suppression of HSC function over TPO-dependent maintenance of LTR activity. Therefore, TPO-induced expression of *Nov* was impaired in HSCs treated with  $IFN\gamma$  (Fig. 5a), which likely mediated the  $IFN\gamma$ -dependent suppressive effects of  $\alpha v\beta 3$  integrin. Under similar conditions, exogenous ligands may give rise to integrin  $\alpha v\beta 3$ -mediated suppression of LTR activity. However because  $IFN\gamma$  seems to influence HSC regulation differently in vivo and in vitro [11, 12], it remains unclear whether the  $IFN\gamma$ -dependent effects of integrin ligands observed in vitro will be recapitulated in vivo.

We also found that *Nov*, but not *VN*, suppressed HSC proliferation in the presence of  $IFN\gamma$  (Figs. 6a, 7a). This suppressive effect of *Nov* was dependent on the presence of  $IFN\gamma$ , as *rmNov* had little effect on proliferation in the presence of TPO alone (Supplemental Fig. 1a). Thus, the differential integrin  $\alpha v\beta 3$ -mediated effects of *Nov* and *VN* on HSC function in the presence of  $IFN\gamma$  may be attributable to their ligand specificity. Unlike *VN*, *Nov* is able to associate with surface antigens other than integrin receptors (e.g., Notch 1 and Connexin 43) [32, 33], an ability that may contribute to its unique effects in the presence of  $IFN\gamma$ . Our results also indicate that *Nov* associates with additional unidentified receptors on HSCs because the binding of *rmNov* to HSCs was not completely compromised by treatment with the anti-integrin antibodies (Fig. 1, Supplemental Fig. 2). These additional *Nov* ligations are not likely to exert a substantial effect on HSC functions because the effects of *rmNov* on HSCs were blocked by either  $\beta 3$  integrin deficiency (Fig. 6e, f) or the impairment of  $\beta 3$  integrin outside-in signaling (Fig. 2b).

In conclusion, we have demonstrated a novel mechanism of *Nov* action on the regulation of HSC function. Our results show that TPO,  $IFN\gamma$  integrin  $\alpha v\beta 3$ , and *Nov* act in concert to regulate HSC function and shed light on the complex regulatory mechanisms governing HSC function within its BM niche. Our findings also expand on the results of our previous study, furthering our understanding of the relationship between integrin and cytokine signaling in HSCs and supporting our previously proposed model of the integrin  $\alpha v\beta 3$ -mediated expansion of HSCs in vitro that does not negatively affect LTR activity [2]. Thus, this study provides a basis for further characterization of the HSC niche and for the development of in vitro methods of HSCs manipulation for transplantation purposes.

## Supplementary Material

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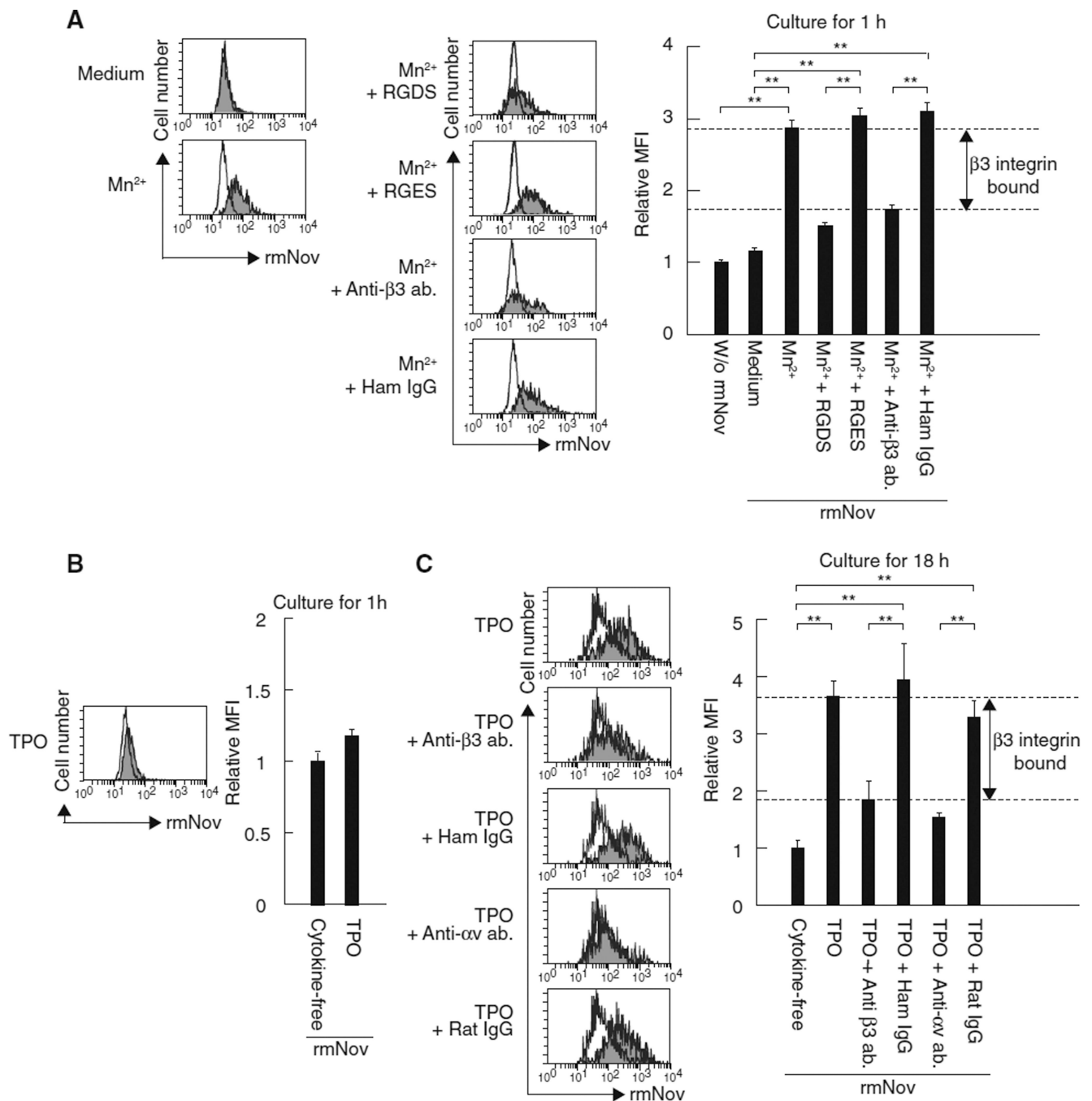
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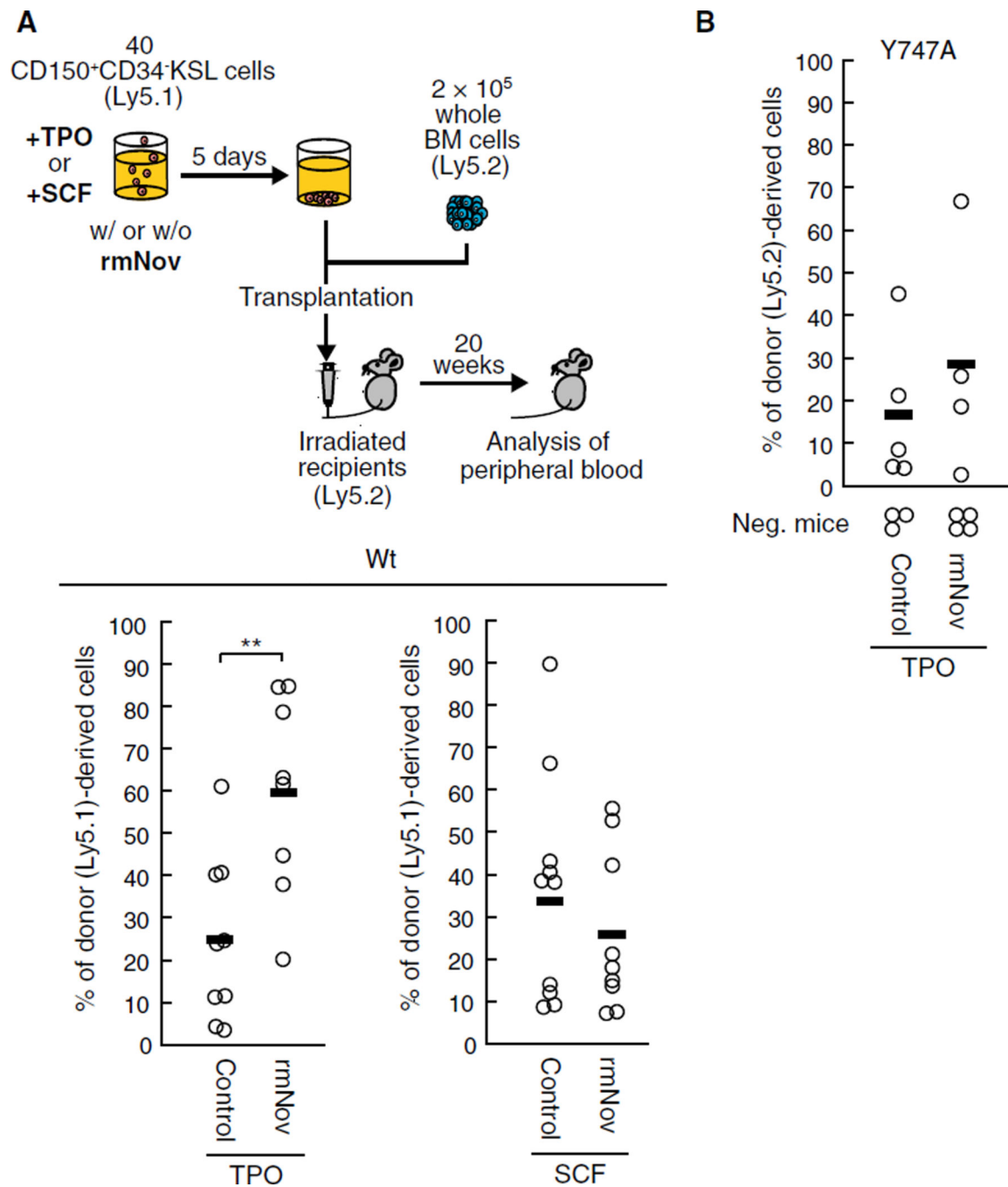
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**Fig. 1.**

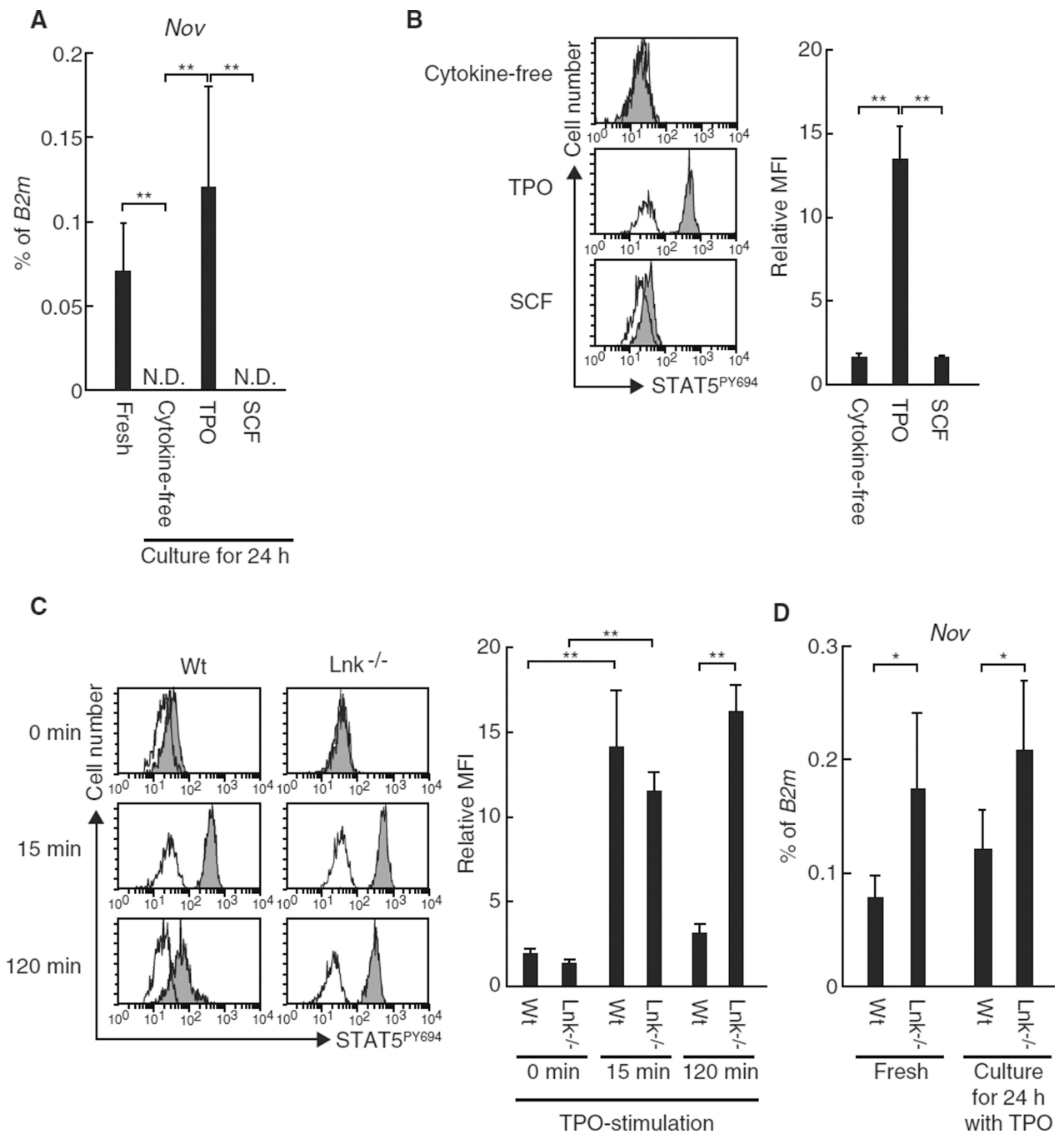
TPO is required for Nov binding to the integrin  $\alpha\beta 3$ . **a** CD34<sup>-</sup>KSL cells were treated for 1 h with or without His-tagged rmNov in the absence or presence of Mn<sup>2+</sup> and subsequently stained with an antibody recognizing the His-tag prior to flow cytometric analyses. For the inhibitory experiments, an RGDS synthetic peptide or an anti- $\beta 3$  integrin antibody was employed. An RGES peptide or Hamster (Ham) IgG served as controls, respectively. The histograms depict the fluorescence intensities of the bound rmNov: *white*, cells cultured in the absence of rmNov; and *gray*, cells treated with rmNov. The graph depicts the relative

mean fluorescence intensity (MFI); the fluorescence intensity in the absence of rmNov served as a control. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 3$ ). In addition, CD34 KSL cells cultured for 1 h (**b**) or 18 h (**c**) were treated with His-tagged rmNov and subsequently treated with an antibody recognizing the His-tag before flow cytometric analyses as described above. Some cells were treated with the indicated antibodies during culture. Ham or Rat IgG was utilized as controls for the anti- $\beta 3$  or  $\alpha v$  integrin antibodies, respectively. The histograms depict the fluorescence intensities of bound rmNov: *white*, cells cultured in the absence of cytokine; and *gray*, cells cultured in the presence of TPO. The graph depicts the relative MFI; the fluorescence intensity in the absence of cytokine served as a control. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 3$ )

**Fig. 2.**

Exogenous Nov enhances LTR activity via the  $\beta 3$  integrin in the presence of TPO. **a** Forty CD150<sup>+</sup>CD34<sup>-</sup>KSL cells isolated from wild type (Wt) mice (Ly5.1) were cultured for 5 days with or without rmNov in the presence of TPO or SCF. The cultured cells were then transplanted into lethally irradiated mice (Ly5.2) along with  $2 \times 10^5$  whole bone marrow (BM) competitor cells (Ly5.2). Twenty weeks after transplantation, the percentage of donor cells (Ly5.1) in the peripheral blood was determined, **b** Forty CD150<sup>+</sup>CD34<sup>-</sup>KSL cells derived from  $\beta 3$  integrin Y747A knock-in mutant mice (Ly5.2) were cultured with or

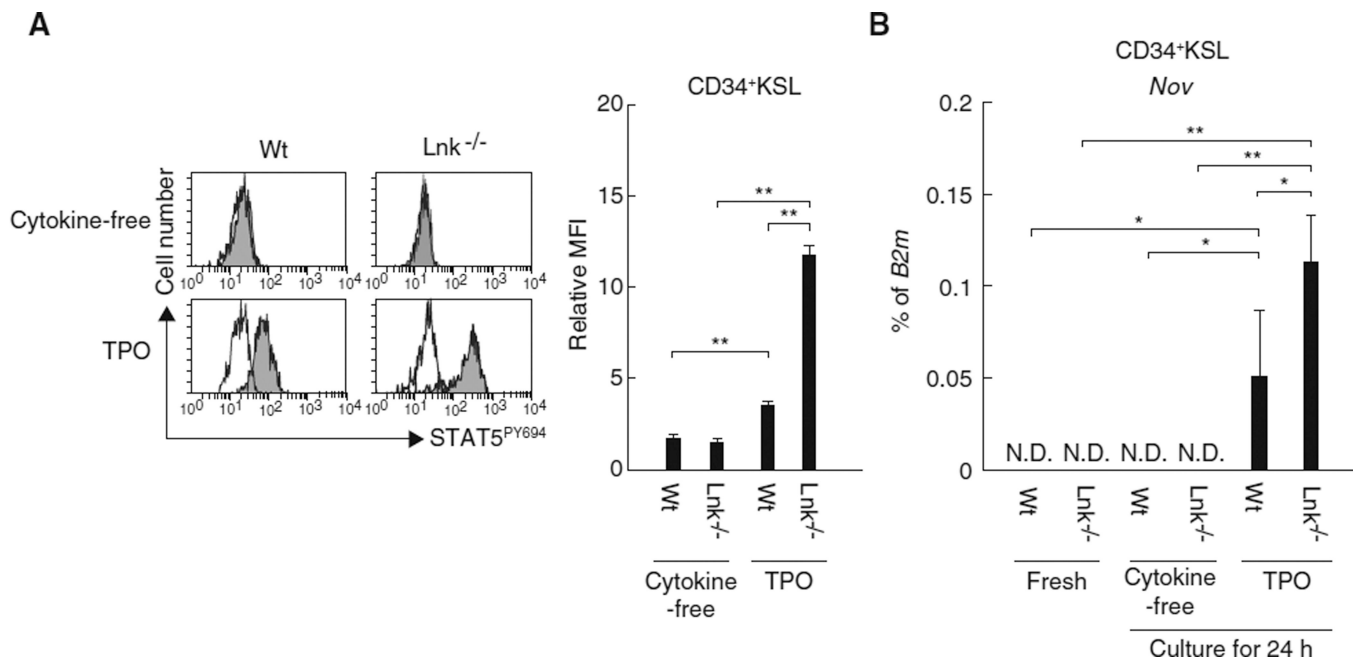
without rmNov in the presence of TPO and subsequently transplanted along with  $2 \times 10^5$  BM competitor cells (Ly5.1) into lethally irradiated recipient mice (Ly5.1) as described above. The *plots* represent the percentage of donor (Ly5.1 or Ly5.2)-derived cells in the peripheral blood of individual mice 20 weeks after transplantation. The *bars* depict the mean values (\*\* $P < 0.01$ ). Recipient mice with donor cell chimerism  $< 1.0\%$  for any lineage were not considered to be reconstituted (negative mice)



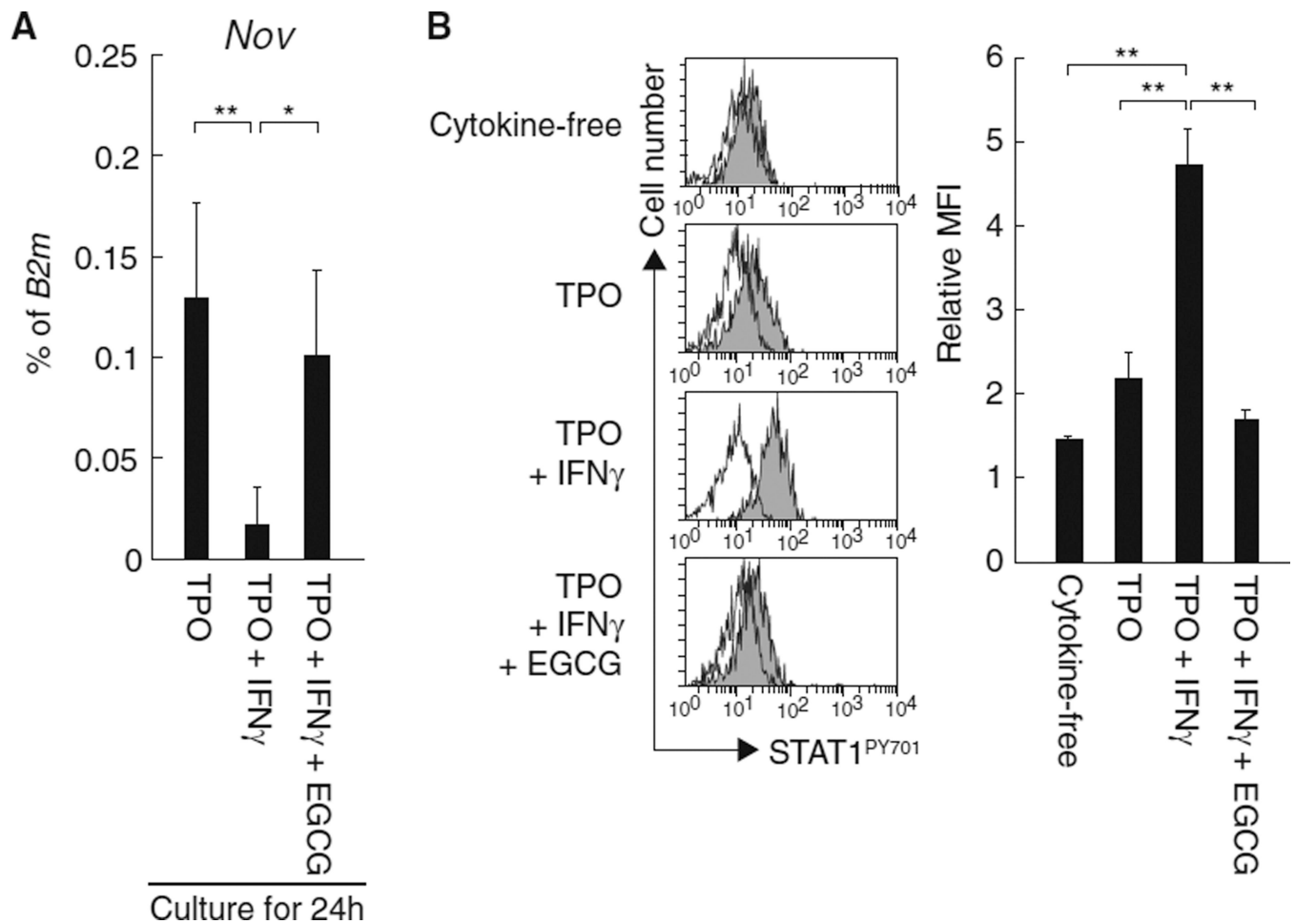
**Fig. 3.** TPO stimulates *Nov* expression in HSCs via STAT5 activation. **a** The expression of *Nov* mRNA was examined using quantitative real-time RT-PCR in uncultured CD150<sup>+</sup>CD34<sup>-</sup>KSL cells (fresh) or in cells cultured for 24 h under the indicated conditions. The graph depicts the levels of *Nov* mRNA expression normalized to the expression of *B2m*. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 5$ ). ND represents samples in which *Nov* expression could not be detected. **b** CD34<sup>-</sup>KSL cells were stimulated for 15 min with the indicated cytokines prior to flow cytometric analyses to examine STAT5 activation. The



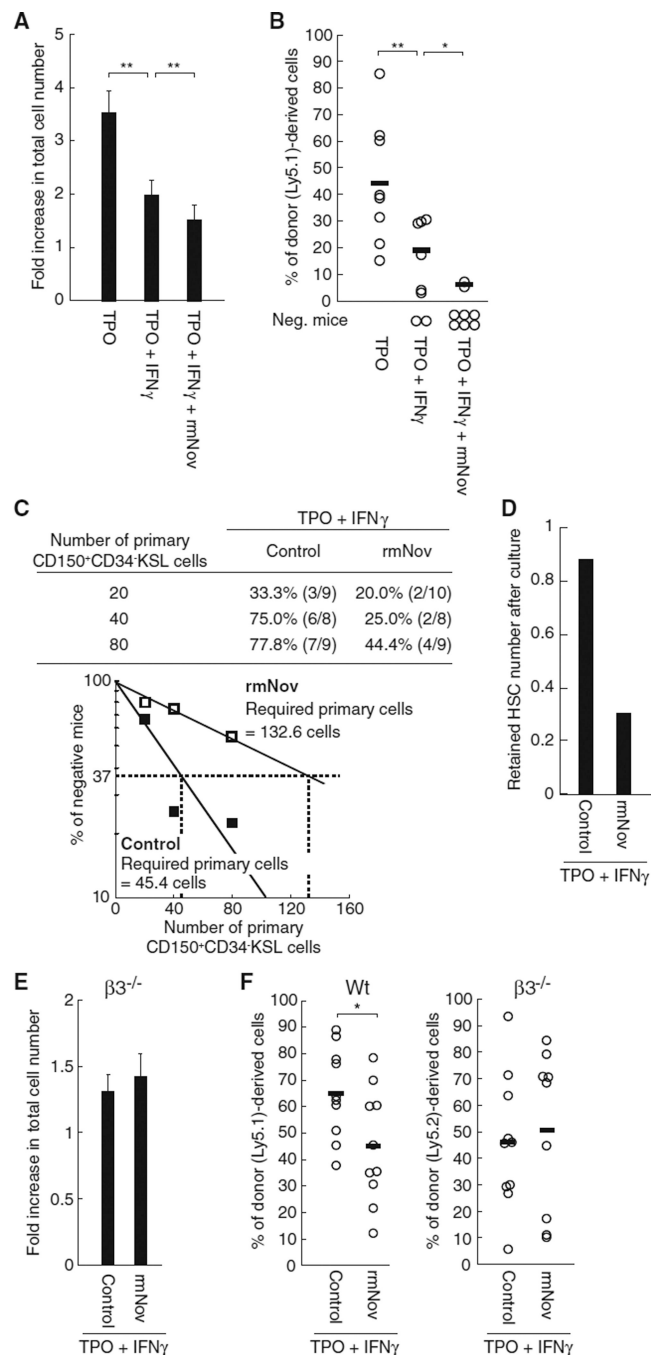
histograms depict the levels of STAT5 phosphorylation at Y694: *white*, isotype control; and *gray*, anti-STAT5<sup>PY694</sup> antibody. The graph depicts the relative mean fluorescence intensity (MFI). Samples stained with control antibodies served as controls for each condition. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 3$ ). **c** CD34 KSL cells derived from wild type (Wt) or Lnk<sup>-/-</sup> mice were stimulated with TPO for the indicated times and STAT5 activation was analyzed by flow cytometry. The histograms represent the levels of STAT5 phosphorylation at Y694: *white*, isotype control; and *gray*, anti-STAT5<sup>PY694</sup> antibody. The graph depicts the relative MFI. Samples stained with isotype control antibody served as the controls for each condition. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 3$ ). **d** CD150<sup>+</sup>CD34<sup>-</sup>KSL cells derived from Wt or Lnk<sup>-/-</sup> mice were subjected to quantitative real-time RT-PCR before (fresh) or after culturing for 24 h in the presence of TPO. The graph depicts the levels of *Nov* mRNA expression normalized to the levels of *B2m* expression. The data represent the mean  $\pm$  SD (\* $P < 0.05$ ,  $n = 5$ )



**Fig. 4.** TPO stimulates *Nov* expression in CD34<sup>+</sup>KSL HPCs. **a** After CD34<sup>+</sup>KSL cells derived from wild type (Wt) or Lnk<sup>-/-</sup> mice were stimulated with TPO for 15 min, the activation of STAT5 was analyzed using a flow cytometer. The histograms represent the levels of STAT5 phosphorylation at Y694: *white*, isotype control; and *gray*, anti-STAT5<sup>PY694</sup> antibody. The graph depicts the relative mean fluorescence intensity (MFI). Samples stained with isotype control antibody served as the controls for each condition. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 3$ ). **b** The expression of *Nov* was analyzed by quantitative real-time RT-PCR in uncultured Wt or Lnk<sup>-/-</sup> CD34<sup>+</sup>KSL cells (fresh) or in cells cultured under the indicated conditions for 24 h. The graph depicts the levels of *Nov* mRNA expression normalized to the levels of *B2m* expression. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ , \* $P < 0.05$ ,  $n = 5$ ). ND represents samples in which *Nov* expression could not be detected

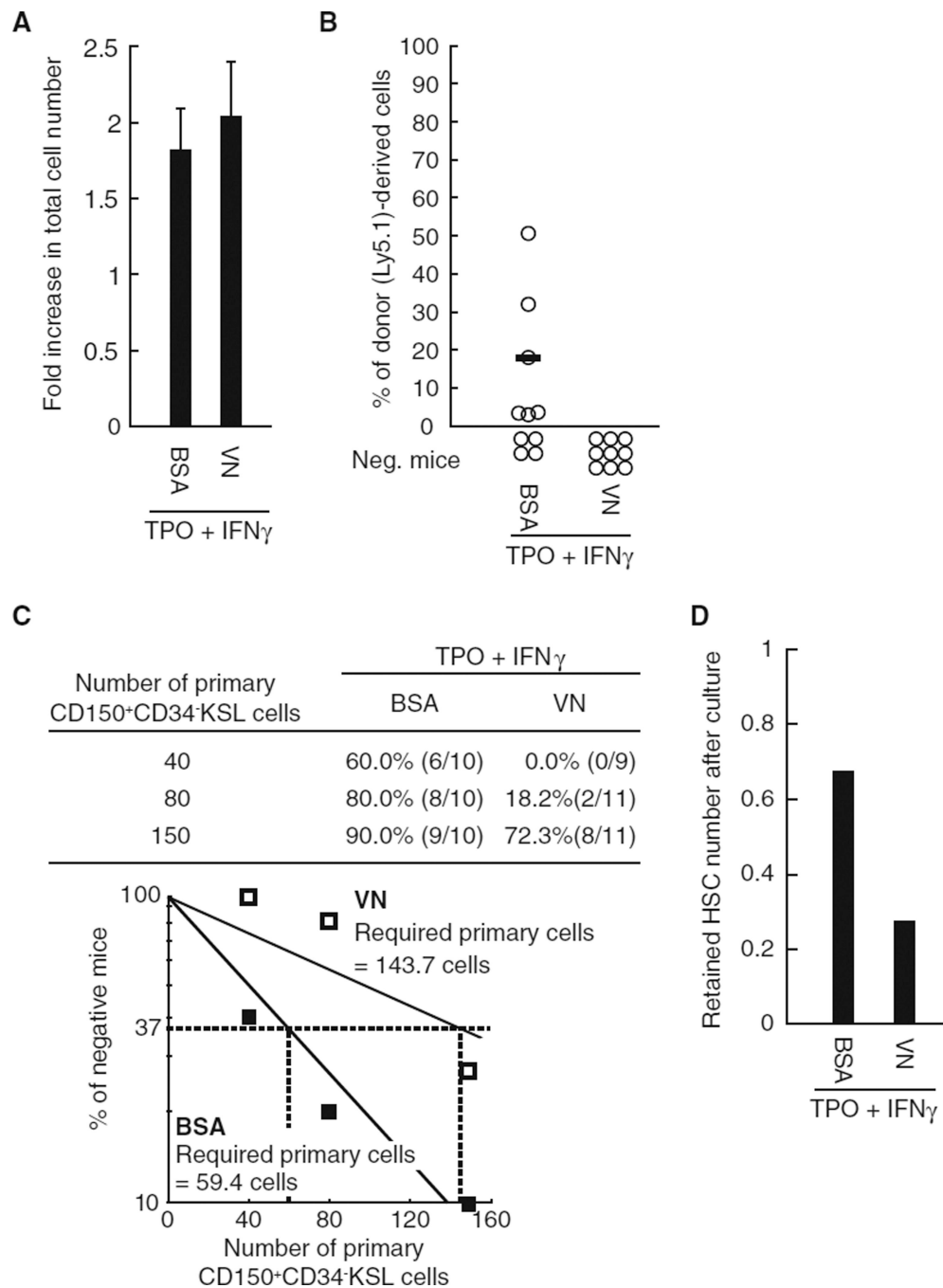


**Fig. 5.** IFN $\gamma$ -dependent activation of STAT1 impairs TPO-stimulated *Nov* expression in HSCs. **a** CD150<sup>+</sup>CD34<sup>-</sup>KSL cells were cultured for 24 h with or without IFN $\gamma$  in the presence of TPO, and the expression of *Nov* mRNA was assessed using quantitative real-time RT-PCR. EGCG is an inhibitor of STAT1 activation. The graph depicts the levels of *Nov* mRNA expression normalized to the levels of *B2m* expression. The data represent the mean  $\pm$  SD (\*\* $P$  < 0.01, \* $P$  < 0.05,  $n$  = 5). **b** CD34 KSL cells were stimulated for 15 min with the indicated cytokines in the presence or absence of EGCG and STAT1 phosphorylation was analyzed by flow cytometry. The histograms depict the levels of STAT1 phosphorylation at Y701: white, isotype control; and gray, anti-STAT1<sup>PY701</sup> antibody. The graph depicts the relative mean fluorescence intensity (MFI). Samples stained with isotype control antibody served as controls for each condition. The data represent the mean  $\pm$  SD (\*\* $P$  < 0.01,  $n$  = 3)



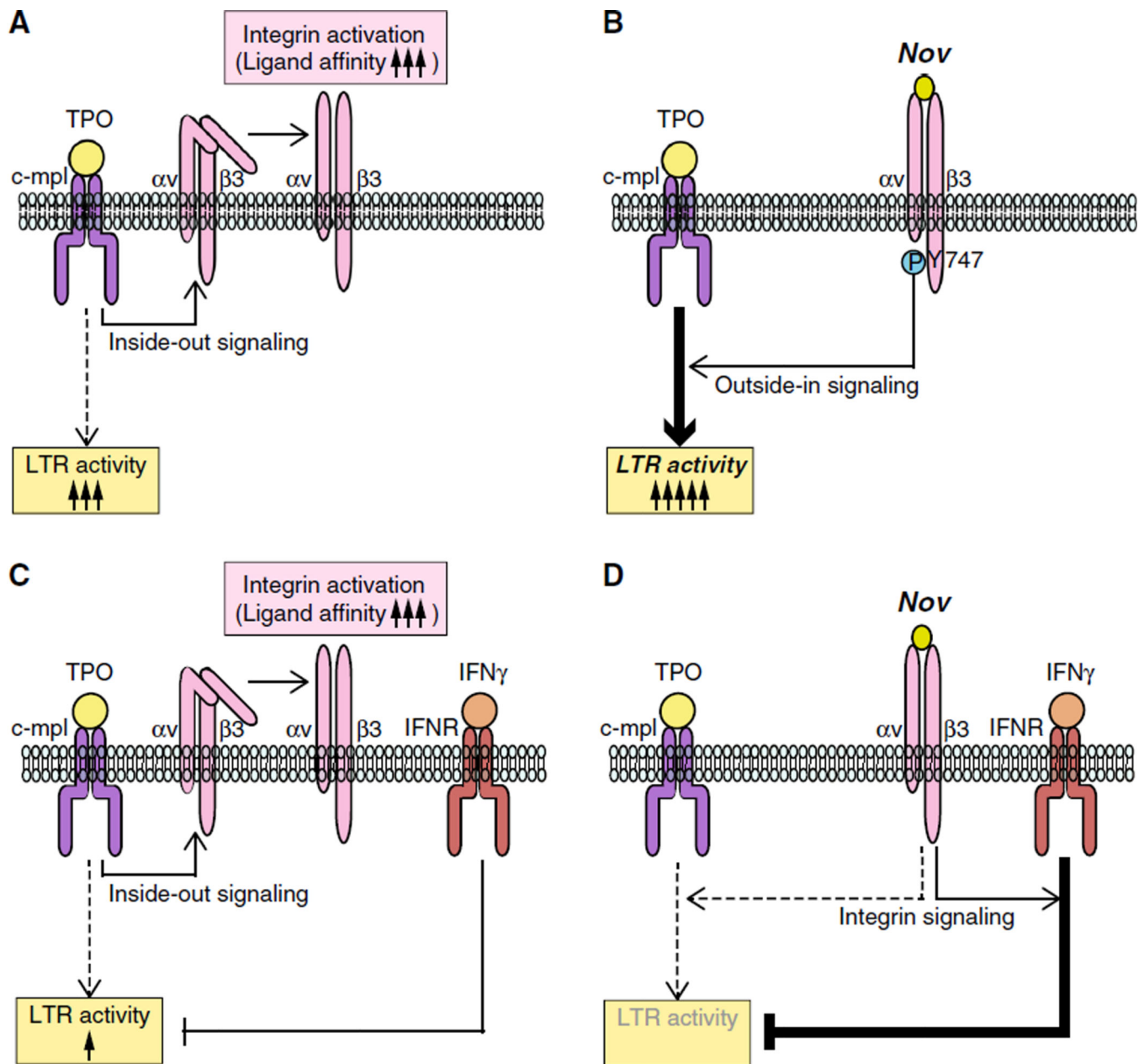
**Fig. 6.** Exogenous Nov acts via  $\beta 3$  integrin to impair the maintenance  $\beta 3$  of LTR activity in the presence of IFN $\gamma$ . Forty CD150<sup>+</sup>CD34<sup>-</sup>KSL cells were cultured in the presence of rmNov, TPO and/or IFN $\gamma$  for 5 days. **a** The total number of cells was quantified. The graph shows the fold-increase in total cell number. The data represent the mean  $\pm$  SD (\*\* $P < 0.01$ ,  $n = 8$ ). **b** The cultured cells were transplanted into lethally irradiated recipient mice along with  $2 \times 10^6$  whole BM competitor cells. The *plots* depict the percentage of donor (Ly5.1)-derived cells in the peripheral blood of individual mice 20 weeks after transplantation. The *bars*

represent the mean values (\*\* $P < 0.01$ , \* $P < 0.05$ ). Recipient mice with donor cell chimerism  $< 1.0\%$  for any lineage were not considered to be reconstituted (negative mice). **c** Groups of 20, 40 or 80 CD150<sup>+</sup>CD34<sup>-</sup>KSL cells were cultured for 5 days with or without rmNov in the presence of TPO and IFN $\gamma$ . Afterward, the groups were individually transplanted as described above. The table shows the percentages of multi-lineage reconstituted mice. The numbers in parentheses denote multilineage reconstituted mice/ tested mice. In addition, the percentages of unreconstructed mice (percentages of negative mice on the  $\gamma$  axis) were plotted versus the numbers of primary CD150<sup>+</sup>CD34<sup>-</sup>KSL cultured cells (*white*, rmNov treatment; and *black*, without rmNov treatment). The estimations of the theoretically required number of primary CD150<sup>+</sup>CD34<sup>-</sup>KSL cells required for retaining one cell with LTR activity after culture under indicated conditions are based on a Poisson distribution, **d** The graph represents the estimated HSC number after culture under the same conditions indicated in Fig. 6b. Two hundred CD150<sup>+</sup>CD34<sup>-</sup>KSL cells isolated from wild type (Wt) (Ly5.1) or integrin  $\beta 3^{-/-}$  mice (Ly5.2) were cultured for 5 days with or without rmNov in the presence of TPO and IFN $\gamma$ . **e** The total number of cells was quantified. The graph shows the fold-increase in the total cell number. The data represent the mean  $\pm$  SD ( $n = 8$ ). **f** The transplantation assays were performed as described above. The plots depict the percentage of donor (Ly5.1 or Ly5.2)-derived cells in the peripheral blood of individual mice 20 weeks after transplantation. The bars represent the mean values (\* $P < 0.05$ )



**Fig. 7.** Vitronectin (VN) impairs the maintenance of LTR activity in the presence of IFN $\gamma$ . Forty CD150<sup>+</sup>CD34<sup>+</sup>KSL cells were cultured for 5 days in the presence of TPO and IFN $\gamma$  on BSA- or VN-coated plates. **a** The total number of cells was quantified. The graph shows the fold-increase in total cell number. The data represent the mean  $\pm$  SD ( $n = 8$ ). **b** In addition, the cultured cells were transplanted into lethally irradiated recipient mice along with  $2 \times 10^5$  whole BM competitor cells. The plots depict the percentage of donor (Ly5.1)-derived cells in the peripheral blood of individual mice 20 weeks after transplantation. The bars represent

mean values. Recipient mice with donor cell chimerism  $<1.0$  for any lineage were not considered to be reconstituted (negative mice). **c** Groups of 40, 80 or 150 sorted  $CD150^+CD34^-KSL$  cells were cultured for 5 days in the presence of TPO and  $IFN\gamma$  on BSA- or VN-coated plates and then used in transplantation assays as described above. The table shows the percentages of multi-lineage reconstituted mice and the numbers in parentheses denote the multilineage reconstituted mice/tested mice. In addition, the percentage of unreconstructed mice (percentage of negative mice on the  $\gamma$  axis) was plotted versus the numbers of primary  $CD150^+CD34^-KSL$  cultured cells (*white*, VN-coat; and *black*, BSA-coat). The estimates of the theoretically required number of primary  $CD150^+CD34^-KSL$  cells needed to retain one cell with LTR activity after culture are based on a Poisson distribution. **d** The graph represents the estimated number of HSCs cultured under the conditions indicated in **b**.

**Fig. 8.**

A model depicting the regulation of Nov/integrin  $\alpha v \beta 3$ -mediated maintenance of LTR activity of HSCs that requires simultaneous stimulation by other cytokines. **a** TPO is not only involved in HSC maintenance, but it also induces  $\alpha v \beta 3$  integrin activation (a conformational change that increases its ligand affinity). **b** Nov associates with the integrin  $\alpha v \beta 3$ , thereby enhancing the TPO-dependent maintenance of LTR activity that is mediated by  $\beta 3$  integrin outside-in signaling, **c** IFN $\gamma$  is capable of impairing the LTR activity of HSCs independently of the TPO-dependent activation of the  $\alpha v \beta 3$  integrin. **d** The presence of exogenous Nov induces  $\beta 3$  integrin-mediated signaling by acting in concert with IFN $\gamma$  rather than via TPO, resulting in further suppression of LTR activity