

Haematologica
HAEMATOL/2016/143958
Version 4

Dual role of IL-21 in megakaryopoiesis and platelet homeostasis

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Disclosures: This work was supported in part by ARMESA.

Contributions: S.B. performed research, designed experiments and analyzed the data and wrote the paper, C.A. and L.M. performed research and analyzed data, M.F. contributed to animal care and handling, C.S. C.G and F.L analyzed the data and reviewed the paper, H.D.L.S. supervised the project, analyzed the data and wrote the paper.

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Dual role of IL-21 in megakaryopoiesis and platelet homeostasis

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Running title: Modulation of platelet homeostasis by IL-21

Acknowledgments:

The authors would like to thank Catherine Ziessel for expert technical assistance, Arnaud Dupuis (Hôpital Universitaire de Strasbourg) for providing human BM samples, Nathalie Brouard for helpful discussions and technical advice and Juliette Mulvihill for reviewing the English of the manuscript.

Abstract

Gene profiling studies have indicated that *in vitro* differentiated human megakaryocytes express the receptor for IL-21 (IL-21R), an immunostimulatory cytokine associated with inflammatory disorders and currently under evaluation in cancer therapy. The aim of this study was to investigate whether IL-21 modulates megakaryopoiesis. We first checked the expression of IL-21 receptor on human BM and *in vitro* differentiated megakaryocytes. Then, we investigated the effect of IL-21 on the *in vitro* differentiation of human blood CD34⁺ progenitors into megakaryocytes. Finally, we analyzed the consequences of hydrodynamic transfection-mediated transient expression of IL-21, on megakaryopoiesis and thrombopoiesis in mice. The IL-21R α chain was expressed in human BM megakaryocytes and was progressively induced during *in vitro* differentiation of human peripheral CD34⁺ progenitors, while the signal transducing γ chain was down-regulated. Consistently, the STAT3 phosphorylation induced by IL-21 diminished during the later stages of megakaryocytic differentiation. *In vitro*, IL-21 increased the number of CFU-MKs generated from CD34⁺ cells and the number of megakaryocytes differentiated from CD34⁺ progenitors in a JAK3- and STAT3-dependent manner. Forced expression of IL-21 in mice increased the density of bi-potent MK progenitors and BM megakaryocytes, and the platelet generation, but increased platelet clearance and consequently resulting in reduced blood platelet counts. Our work suggests that IL-21 regulates megakaryocyte development and platelet homeostasis. Thus IL-21 may link immune responses to physiological or pathological platelet-dependent processes.

INTRODUCTION

Megakaryopoiesis is mainly controlled by thrombopoietin (TPO). *In vitro*, TPO is essential to differentiate hematopoietic progenitors into MKs, a differentiation enhanced by cytokines such as IL-6, IL-1 β , IL-3, IL-9 and IL-11¹. *In vivo*, megakaryopoiesis occurs in the bone marrow (BM), a complex environment in which innate and adaptive immune cells produce cytokines regulating this process, some positively –such as IL-6, TNF- α and IL-1 β ^{2, 3} others negatively –IL-10, IL-4 and TGF- β ⁴⁻⁶. This influence is exemplified by reactive thrombocytosis, attributed to infections or inflammatory diseases⁷ and largely mediated by IL-6⁸. More recently, IL-1 α was shown to enhance thrombopoiesis⁹. Whether other cytokines are involved in the regulation of megakaryopoiesis requires further investigations in order to better understand inflammatory thrombocytosis. To address this issue, we compared published gene profiles of CD34⁺ progenitors and, *in vitro* differentiated megakaryoblasts¹⁰. This analysis indicated the presence of IL-21 receptor (IL-21R) on megakaryocytes.

IL-21R is a heterodimer composed of a specific alpha chain (IL-21R α) and the common gamma chain (IL-2R γ) required for signal transduction¹¹. IL-21 is produced by subsets of NKT cells and helper CD4⁺ T cells, in particular follicular Th cells and Th17 cells. In healthy individuals, the BM contains T cells producing IL-21¹², likely follicular Th cells whose frequency may increase in pathological states¹³. IL-21 controls a variety of responses of different immune cells such as B, NK and T lymphocytes, macrophages and dendritic cells and also vascular endothelial cells¹¹. IL-21 is associated with the development of autoimmune diseases and inflammatory disorders and hence, like IL-6, could play a role in reactive thrombocytosis. Notably, IL-6 induces the production of IL-21 by CD4⁺ T cells. Recently, IL-21 was found to be expressed by mouse hematopoietic stem cells and progenitors when stimulated by TLR activators released by apoptotic cells¹⁴.

The aim of this study was to investigate the role of IL-21 on megakaryopoiesis, using *in vitro* assays with human cells and *in vivo* experiments in mice.

METHODS

Techniques are described in details in the Online Supplementary Information.

Antibodies. Mouse anti-human CD41/CD61, CD42b and CD42d and rat anti-mouse and human CD42c antibodies were prepared in our laboratory, other antibodies were commercial.

In vitro differentiation of human MKs.

Peripheral CD34⁺ progenitors from healthy blood donors were isolated and cultured in serum free media and appropriate cytokine combinations.

CFU-MK assays. CFU-MKs assays were performed using MegaCult™-C Kits with cytokines (StemCell Technologies) according to the manufacturer's instructions. Three independent experiments were performed in quadruplicate.

Quantification of proplatelet-bearing human MKs. On day 13 of culture, images of ten different wide fields in 24 well plates were acquired using an inverted microscope coupled to a camera (Zeiss). Round and proplatelet-bearing MKs were counted.

RT-PCR analyses. Semi-quantitative RT-PCRs were performed using total RNA of CD34⁺ progenitors and cultured CD41/CD61⁺ cells. The identity of the RT-PCR products was confirmed by DNA sequencing.

Immunofluorescence microscopy. Human BM specimens from the iliac crest were obtained from individuals having a normal megakaryocytic lineage. Mouse femora, spleens and livers were harvested, fixed then decalcified (femora). Samples were embedded in OCT compound and cryosectioned at 8 µm. On day 13 of culture, megakaryocytes were fixed and cytospun onto poly L-lysine-coated slides. IL-21Rα was revealed using a tyramide amplification technique (TSA PLUS Fluorescence Kit, Perkin Elmer). MKs and macrophages were counterstained with anti-CD42c or F4/80 antibody, respectively, before analysis by confocal microscopy (TCS SP5, Leica Microsystems).

Flow cytometry (FC).

Cells were labelled as described in the Online Supplementary Information and analyzed on a Gallios or a BD LSRFortessa cytometer, data were analyzed with Kaluza (Beckman Coulter) or FACSDiva (BD Biosciences) software, respectively.

Hydrodynamic transfections. Murine IL-21 cDNA was cloned into the pLIVE expression vector (Mirus Bio LLC). Empty or recombinant plasmids were intravenously injected into mice.¹⁵ Plasma samples were stored at -80°C before quantification of IL-21 concentration.

Mouse platelets. The percentage of reticulated platelets was checked by FC after staining with Thiazole Orange (TO) and anti-CD42c mAb. To measure platelet survival, washed EGFP⁺ platelets were retro-orbitally injected into mice 5 days after hydrodynamic transfection. The ratio of EGFP⁺ transfused to EGFP⁻ endogenous platelets was determined by FC.

Statistics. All values are reported as the mean \pm SEM. Statistical analyses were performed with GraphPad software (Prism Version 5.0) using Student's t test, or 1-way or 2-way ANOVA and a Bonferroni post-test.

Results

Human MKs express the IL-21 receptor

Peripheral blood CD34⁺ progenitors were differentiated into MKs using a two phase protocol optimized to generate large numbers of MKs: firstly, 7 days of culture in the presence of TPO, IL-6, IL-9 and SCF which allows the expansion of MK progenitors and secondly, 6 days of culture in the presence of TPO alone, to generate mature MKs. RNA was extracted from freshly isolated CD34⁺ cells and from purified CD41/CD61⁺ cells, isolated on days 4, 7 and 10 of culture (Fig. 1A, Scheme 1, without IL-21). Semi-quantitative RT-PCR analyses showed that *IL21R* was not expressed in freshly isolated CD34⁺ cells but was progressively induced during their megakaryocytic differentiation. In contrast, *IL2RG* transcripts were detected in the progenitor cells, their numbers relatively increased during the first days of culture, and

then decreased (Figure 1B). FC analysis of *in vitro* differentiated MKs confirmed the progression of IL-21R α chain expression on CD41/CD61⁺ cells (Figure 1C). In human BM samples, IL-21R α was expressed on a subpopulation of mature megakaryocytes identifiable owing to their large size (> 20 μ m) and CD42c and DAPI staining (Figure 1D). Among 211 MKs from 3 individuals, 37 \pm 7% expressed IL-21R.

The final steps of megakaryopoiesis are characterized by proplatelet formation followed by platelet release. At day 13 of culture, fixed and cytopun MKs were analyzed by immunofluorescence microscopy after labeling with anti-CD42c and anti-IL-21R mAbs (Online Supplementary Figure 1A). IL-21R α was detected on the cellular body of about 65% of the cells but not on proplatelets. Accordingly, IL-21R α could not be revealed on the surface of human blood platelets, in the resting state as previously described¹⁶ or after thrombin-stimulation, nor intracellularly (Online Supplementary Figure 1B).

Altogether, these results confirm the expression of IL-21 receptor on human megakaryocytes and suggest a role of IL-21 during megakaryopoiesis but not in platelet functions.

IL-21R activity changes during the *in vitro* differentiation of MKs

In order to check the functionality of IL-21R in CD34⁺ cell-derived MKs, we focused on STAT3 and STAT5 phosphorylation, which occurs after stimulation of T cells by IL-21¹¹ and of MKs by TPO¹⁷. At days 7 and 12 of MK differentiation, corresponding to the ends of the two culture phases, the cells were starved of cytokines for 5 h, then incubated for 15 min with IL-21 and/or TPO, before fixation and immunostaining with anti-CD41/CD61, -pSTAT3 and -pSTAT5 antibodies. FC analysis revealed that IL-21 induced the phosphorylation of only STAT3 in CD41/CD61⁺ cells, the ratio of responding cells being higher on day 7 (46 \pm 4.9%, n=3) than on day 12 (15 \pm 2.9%, n=3) (Figure 2). TPO induced mainly STAT5 phosphorylation on day 7 of culture, and both STAT3 and STAT5 on day 12. On day 7, IL-21 and TPO signaling were additive. To confirm that STAT3 phosphorylation was mediated by IL-21R, we used the neutralizing anti-IL-21R recombinant monoclonal antibody ATR-107¹⁸.

As expected, this antibody completely inhibited the IL-21-induced phosphorylation of STAT3 in CD41⁺ cells at day 7 of culture, when signal transduction was maximal under our experimental conditions (Online Supplementary Figure 2).

Thus, IL-21R is functional during *in vitro* differentiation of MKs but, probably due to down-regulation of the γ chain, tends to lose its functionality during their maturation.

IL-21 promotes the *in vitro* proliferation of MK progenitors through the JAK3/STAT3 pathway

We first measured the effect of IL-21 on the megakaryocytic potential of CD34⁺ progenitor cells in collagen-based medium containing TPO, IL-3 and IL-6. IL-21 increased the number of medium sized (21 to 50 cells per colony) and large colonies (> 50 cells per colony), on an average by approximately 30% and 90%, respectively (n=3) (Figure 3A). We then investigated the effects of IL-21 on the *in vitro* differentiation of CD34⁺ progenitors into MKs. When blood CD34⁺ cells were cultured for 7 days in the presence of TPO alone (Figure 1A, scheme 2), addition of IL-21 increased the total number of viable cells by 50% on an average and doubled the number of CD41⁺ cells (Figure 3B-D). Specificity of the IL-21-induced cellular responses was confirmed by blocking IL-21R with anti-IL-21R antibody ATR-107: addition of this antibody on days 1 and 3 completely blocked IL-21-induced increase in the yield of the total number of cells and the percentage of CD41⁺ cells obtained at day 7 (Figure 3E). IL-6 in combination with soluble IL-6R has been shown to increase the yield of the *in vitro* differentiation of CD34⁺ progenitors into MKs in the presence of TPO alone¹⁹. IL-21 increased this effect without impacting the ratio of CD41⁺, CD42⁺ or GPV⁺ cells as assessed in 7 day culture assays (Figure 3F and data not shown). However, when CD34⁺ cells were cultured in the presence of the Megakaryocyte Expansion Supplement cocktail (Figure 1A, scheme 3, CC220), addition of IL-21 in the first step did not improve the number of viable cells and of CD41⁺ cells obtained at day 7 (data not shown).

In order to obtain larger numbers of MK progenitors, CD34⁺ cells were differentiated in the presence of CC220 cocktail according to cell culture scheme 1. Cells obtained after 7 days of culture were cultured in the presence of TPO for 6 additional days in the presence of increasing concentrations of IL-21. Under these experimental conditions, IL-21 increased the number of MKs in a dose dependent manner, reaching 1.9 ± 0.1 fold increase at 100 ng/mL (n=6) (Figure 3G). The cell viability was not modified by the presence of IL-21 (data not shown).

Among the Janus kinases, JAK3 is the only molecule linked to IL-2R γ , mediating signal transduction via STAT3 phosphorylation. Since IL-21 induced STAT3 phosphorylation in differentiating MKs, we checked whether its effect on MK generation depended on the JAK3/STAT3 pathway. CD34⁺ progenitors were cultured according to scheme 1; on day 7, several concentrations of Tofacitinib (a JAK3 inhibitor) and Stattic (a STAT3 inhibitor), or their vehicle (DMSO, 0.25%), were added to the cultures, supplemented or not with IL-21 (100 ng/ml). Analysis of cells 5 days later revealed that 300 nM Stattic or 1500 nM Tofacitinib had no impact on the number of MKs obtained in the presence of TPO and the vehicle (DMSO) (Figure 3H). The combination of TPO + IL-21 afforded a 1.5 ± 0.03 (n=5) fold increase in the number of MKs, as compared to TPO with the vehicle. Addition of Stattic or Tofacitinib, abolished this enhancement (1.16 ± 0.03 and 1.06 ± 0.05 fold respectively, n=5). FC analysis revealed that the inhibitors, at these concentrations, did not affect the differentiation of MKs since in all conditions, 90% of the cells were CD41⁺ CD42⁺ (data not shown). Altogether, these results indicate that IL-21 promotes the *in vitro* proliferation of MK progenitors through the JAK3/STAT3 pathway.

MK phenotype and platelet production are not modified by IL-21

At the end of the second phase of culture (scheme 1), we evaluated the influence of IL-21 on the MK phenotype (Online Supplementary Figure 3). IL-21 did not significantly affect the surface expression of CD41, CD42c or CD42d on viable cells, their ploidy, the proportion of

proplatelet-bearing MKs, or the number of platelets released per MK. Thus, IL-21 had no obvious effect on the phenotype of mature MKs or on platelet production.

IL-21 stimulates megakaryopoiesis *in vivo*

To appraise the *in vivo* relevance of these observations, additional experiments were performed in mice. Firstly, we confirmed that IL-21 increased the number of MKs differentiated *in vitro* from murine BM Lin⁻ cells in the presence of TPO (1.4 ± 0.15 fold increase, $n=5$), without affecting the phenotype of the cells (expression of CD41/CD61 heterodimer and CD42c, and polyploidy, Online Supplementary Figure 4). This positive effect could be inhibited by the ATR-107 mAb, which also antagonizes mouse IL-21R. IL-21 was then transiently expressed in mice using hydrodynamic transfection of an IL-21 expression vector (pLIVE-IL-21). In negative control mice receiving the empty vector (pLIVE), the plasma concentration of IL-21 remained below the detection limit (<64 pg/mL). In contrast, one day after transfection of pLIVE-IL-21, the plasma concentration of IL-21 reached 3227 ± 952 pg/mL, then progressively declined to 494 ± 97 pg/mL at day 7, reaching 124 ± 17 pg/mL by day 16 post transfection ($n=7$) (Online Supplementary Figure 5). Animals were analyzed during the first 7 days following the hydrodynamic transfer.

We first analyzed the numbers of MK progenitors in the BM of the transfected mice on day 6. In the mouse, these cells belong to the Lin⁻Sca⁻IL7R α ⁻cKit⁺CD150⁺CD16/32^{lo} population. Within this subset, CD9^{hi}CD105^{lo} and CD9^{lo}CD105^{hi} cells represent megakaryocyte (PreMK) and erythroid (PreE) precursors respectively, while CD9^{lo}CD105^{lo} cells are bipotent erythroid-megakaryocyte progenitors (BEMP)²⁰. The percentages of PreEs and BEMPs were significantly increased 6 days in pLIVE-IL-21 transfected animals, while PreMKs were not significantly expanded (Figure 4A). In the spleen, an important secondary hematopoietic organ, the Lin⁻Sca⁻IL7R α ⁻cKit⁺CD150⁺CD16/32^{lo} progenitors appeared to be rarer, nevertheless their number was increased by IL-21 expression (Figure 4B).

To complete the FC analysis, we compared BM and spleen cells in CFU-MK assays using a collagen-based medium containing TPO, IL-3 and IL-6. These assays showed that the forced

expression of IL-21 resulted in increased numbers of CFUs of MK-containing mixed colonies and non-MK colonies in the BM and the spleen, while numbers of CFUs of pure MK colonies were increased only in the spleen (Figure 4C). The ratios of CFU-MKs (pure and mixed) to the number of seeded cells from control and IL-21 expressing mice were approximately 47- and 28-higher for BM than for spleen cells, respectively, indicating the rarity of megakaryocyte progenitors in the spleen and suggesting a minor contribution of spleen to megakaryopoiesis under these experimental conditions.

Finally, immunofluorescence analysis of BM sections stained with an anti-CD42c mAb revealed an increased MK density in mice expressing IL-21 as compared to control animals (160 ± 8 vs. 129 ± 4 MKs per mm^2 respectively, $n=6$) (Figure 4D).

Altogether, these data showed that IL-21 expression increases the number of MK progenitors in the BM and the spleen and enhances megakaryopoiesis, confirming the *in vivo* relevance of our *in vitro* observations.

IL-21 expression increases platelet clearance

One day after hydrodynamic transfection, total platelet counts decreased by 50% (Figure 5A) and a similar decrease was observed after injection of the transfer solution or saline alone (data not shown). Thereafter, in pLIVE-receiving control animals platelet counts progressively increased to recover their baseline value 6 days later ($n=6$) (Figure 5A, left diagram). Conversely, in pLIVE-IL-21 transfected mice, platelet counts unexpectedly remained reduced. However, in agreement with the observed higher MK density in the BM, the number of $\text{TO}^{\text{bright}}$ young platelets was increased, compared to control mice (Figure 5A, middle and right diagrams). On day 6, the phenotype of the platelets from pLIVE and pLIVE-IL-21 transfected mice and their responses to thromboxane and PAR4 agonists were similar; the responses of youngest ($\text{TO}^{\text{bright}}$) and older (TO^{dim}) platelets to agonists were also identical (Online Supplementary Figure 6).

This prolonged thrombocytopenia in mice transfected with pLIVE-IL21 could result from a peripheral mechanism, probably an increased clearance. To test this hypothesis, washed EGFP⁺ platelets from untreated mice were transfused into untransfected, control transfected or pLIVE-IL-21 transfected EGFP⁻ mice and their counts were followed for 5 days. Two days after transfusion, the percentage of circulating EGFP⁺ platelets was lower in mice expressing IL-21 (n=6) as compared to control transfected (n=7) or untransfected animals (n=6) ($17 \pm 2\%$ vs. $45 \pm 2\%$, $P < 0.001$, and vs. $52 \pm 1\%$, $P < 0.001$, respectively) (Figure 5B), confirming that IL-21 expression increased platelet clearance.

Since the spleen can retain a pool of platelets which increases with splenomegaly²¹, we weighed the spleens and found that 6 days after transfection, the spleens of mice expressing IL-21 were larger than those of control animals (163 ± 8 mg vs. 88 ± 6 mg respectively, n=9) (Figure 5C, Spleens panel, left). Additional experiments showed that splenomegaly was already maximal by day 3 and stable for at least 9 additional days (data not shown). The distribution of macrophages and platelets in the spleen were analyzed by immunofluorescence fluorescence microscopy (Figure 5C, Spleens panel, right and Online Supplementary Figure 7A). The areas of F4/80+ macrophages in spleen cryosections from control mice and animals expressing IL-21 were similar. The area of platelets (CD42c⁺) was slightly but significantly increased in mice expressing IL-21 (Figure 5C, Spleens panel, middle), indicating an increased accumulation of platelets.

To confirm the participation of the spleen in IL-21-induced persistent thrombocytopenia, complementary experiments were performed with splenectomized mice. In these animals, IL-21 expression also increased the number of circulating TO^{bright} platelets (Figure 5D), confirming that IL-21 enhanced BM megakaryopoiesis. Nevertheless, total platelet counts increased similarly in mice expressing IL-21 and control animals, with $1802 \pm 63 \times 10^3$ (n=7) vs $1755 \pm 78 \times 10^3$ (n=6) platelets/ μ L respectively, 5 days after transfection. This latter observation, together with the findings in non splenectomized animals, indicated that the spleen participates in IL-21-induced platelet clearance.

In hydrodynamically transfected splenectomized mice, control mice and those expressing IL-21 displayed similar total platelet counts, despite the increased TO^{bright} platelets, suggesting increased platelet clearance by liver macrophages. We thus also analyzed the livers in transfected animals. IL-21 expression did not impact the weight of the livers, but significantly increased the numbers of macrophages and platelets present in this tissue (Figure 5C, Livers panel and, Online Supplementary Figure 7B). Altogether, these observations suggest that macrophages in the spleen and the liver could be involved in the increase platelet clearance mediated by IL-21.

DISCUSSION

We here showed that IL-21R is expressed when human CD34⁺ progenitors are cultured in the presence of TPO. The receptor was expressed on megakaryocytic CD41⁺ cells, but not on CD41⁻ cells (Figure 1C). Similar observations have likewise been described^{10, 16}. TPO alone also induced the expression of IL-21R on 40-50% of CD41⁺ within 7 days of culture (data not shown). The progressive IL-21R α expression during MK differentiation and its presence on human BM MKs strongly suggest that this expression results from commitment of the progenitor cells to the megakaryocytic lineage. On the other hand, IL-21 increased the numbers of medium and large MK colonies obtained in CFU-MK assays with blood CD34⁺ progenitors, which suggests that this cytokine could also promote the expansion of committed cells.

IL-21R α was not detected on human and mouse platelets. Another example of discordance between MKs and platelets in the expression of a membrane-associated protein is the tyrosine phosphatase receptor CD45, which is likewise present on human BM MKs but not on platelets²². IL-21 induced the phosphorylation of STAT3 in CD41⁺ cells, the percentage of responding cells being higher on day 7 than on day 12, in agreement with the down-regulation of the common γ chain. TPO induced the phosphorylation of only STAT5 on day 7 of *in vitro* human MK differentiation, but both STAT3 and STAT5 phosphorylation was

observed during the later stages of differentiation. This finding is consistent with studies documenting the TPO-induced phosphorylation of STAT3 and STAT5 in megakaryocytic cell lines^{23, 24} and blood platelets^{17, 25}. Thus, at late stages of MK differentiation, the down-regulation of IL-21R-mediated signaling could be compensated by TPO activity. *In vitro*, in the presence of TPO alone, IL-21 increased in a dose dependent manner the number of megakaryocytic cells generated. This effect was inhibited using the blocking anti-IL21R ATR-107 mAb and was dependent on IL-21R-mediated activation of JAK3/STAT3 pathway. These observations are in line with the documented role of STAT3 in megakaryopoiesis^{26, 27}. In the presence of TPO, IL-21 increased the capacity of IL-6 and soluble IL-6R combination to improve the *in vitro* differentiation of CD34⁺ cells into MKs, indicating complementary physiologic functions.

IL-21 and IL-21R deficiencies cause immunodeficiency syndromes in man,^{28, 29} disturb B, T and NK lymphocyte functions in mice¹¹ but do not impact platelet counts, indicating that IL-21 is dispensable for megakaryopoiesis and thrombopoiesis under healthy conditions. Our observations suggest that IL-21 could compensate for increased platelet clearance in pathologies characterized by an increased production of IL-21. Thus, a hydrodynamic transfection method was used to overexpress IL-21 *in vivo*, in hepatocytes.¹⁵ Plasmatic IL-21 concentrations were enhanced for 2 weeks, while IL-1 α , IL-1 β and IL-6 remained undetectable between day 1, and 7 (i.e. < 16pg/ml), in agreement with previous studies,^{30, 31} and no significant TPO variations were noticed (pLIVE vs pLIVE-IL-21 transfected animals, n=3/day, data not shown).

In IL-21-expressing mice, among the BM Lin⁻Sca⁻IL7R α ⁻cKit⁺CD150⁺CD16/32^{lo} progenitors, the ratio of BEMPs were expanded. Consistently, clonogenic assays revealed that IL-21 enhanced the number of megakaryocytic mixed colonies obtained from BM cells. MK are thought to derive from PreMKs, which differentiate from BEMPs³². Because the density of BM MKs was increased, the observed numbers of BEMPs and PreMKs may reflect differences in the dynamics of the maturation of these two types of progenitors.

In the spleen, the numbers of Lin⁻Sca⁻IL7R α ⁻cKit⁺CD150⁺ CD16/32^{lo} progenitors and of pure and mixed CFU-MK were increased by IL-21 expression. The number of CFU-MK per seeded cell was 30 to 50 fold lower in the spleen than in the BM, suggesting that in our experimental conditions, IL-21 has a minor effect on spleen megakaryopoiesis. Our results are in agreement with and complement previous work, where splenomegaly and increased numbers of Lin⁻cKit⁺Sca1⁺ hematopoietic stem cells and CFU-GEMMs (granulocyte-erythrocyte-megakaryocyte-macrophages) were observed in the BM and spleen of mice expressing IL-21 after hydrodynamic transfection³³.

Accordingly, thrombopoiesis was enhanced in transfected mice expressing IL-21, as indicated by their increased counts of young reticulated platelets. However, total platelet counts did not follow this trend. The day after hydrodynamic transfection thrombocytopenia was noticed, probably due to vascular damage and consequently, platelet consumption¹⁵. Normal platelet count was restored between days 4 and 7 in mice transfected with the empty plasmid, but not in pLIVE-IL-21 transfected animals. An increased clearance of platelets could explain this absence of recovery (Figure 5A and B). On day 6, greater numbers of macrophages and platelets were found in the liver of mice expressing IL-21. On cryosections of the enlarged spleen of these animals, macrophages and platelets occupied similar and higher surface areas, respectively (Figure 5C). Thus, increased clearance of platelets could occur in the spleen, or in the liver, another major site for the elimination of old or activated platelets³⁴. IL-21 expression in splenectomized animals was accompanied with increased thrombopoiesis, but no thrombocytopenia, indicating that (i) IL-21-mediated enhanced megakaryopoiesis mainly occurs in the BM and (ii), that a major part of the thrombocytopenia provoked by IL-21 expression is mediated by the spleen. The absence of increased platelet counts in IL-21 splenectomized animals, although thrombopoiesis is increased could be explained by increased clearance by liver macrophages, since IL-21 expression increased the density of platelets and macrophages in this organ. Thus, additional investigations are necessary to elucidate the mechanisms responsible for the increased platelet clearance induced by IL-21.

This study in the mouse is reminiscent of a preclinical study in macaques, which periodically received non-glycosylated recombinant IL-21 and experienced cycles of moderate anemia and thrombocytopenia followed by polycythemia and thrombocytopenia³¹. Because the clearance of the recombinant IL-21 was rapid, it is not possible to compare these observations with ours, which are based on the *in vivo* expression of IL-21. Of note, cancer patients receiving IL-21 could also develop reversible thrombocytopenia³⁵. It is tempting to speculate that in humans receiving recombinant IL-21, megakaryopoiesis and platelet production are increased, but platelet clearance is also enhanced and the net result is thrombocytopenia.

This study demonstrates that IL-21R is progressively induced during human megakaryopoiesis. IL-21 appears to enhance the proliferation of MK progenitors through the JAK3/STAT3 pathway, resulting in increased *in vivo* megakaryopoiesis. Since IL-21 is mainly produced by T cells, our work provides new insights into the regulation of megakaryopoiesis through adaptive immunity. Our observations also suggest that the IL-21-mediated increased megakaryopoiesis could be a compensatory mechanism to counteract enhanced platelet clearance during immune responses characterized by increased IL-21 expression.

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Figure legends

Figure 1. Human *in vitro* differentiated and BM MKs express the IL-21 receptor. (A) Human CD34⁺ cells were isolated from adult peripheral blood and cultured in a serum-free medium, in the presence of Megakaryocyte Expansion Supplement (CC220 cocktail, containing TPO, SCF, IL-6 and IL-9) for the first 7 day phase and of TPO alone for the second 6 day phase. (B) Gel electrophoresis analysis of the RT-PCR products of *IL2RG* and *IL21R* mRNA and 18S rRNA (internal control) extracted from freshly isolated CD34⁺ cells (day 0) and CD41/CD61⁺ sorted cells on days 4, 7 and 10 of culture. A representative gel from three independent experiments is shown. RT₀ is the negative control without cDNA. (C) Cells were labeled with anti-CD41/CD61-Alexa 488, -IL-21R α -APC antibodies, 7AAD and analyzed by FC. Representative FC dot plot analyses of the CD41/CD61 and IL-21R α distribution among live cells (7AAD⁻) from day 0, 4, 7 and 10 cultures are shown. The percentages of live cells are depicted in each quadrant as the mean \pm SEM in three independent experiments. (D) Human BM samples were fixed and labeled with an anti-IL-21R α antibody, stained with FITC using a tyramide-based amplification method (green) and counterstained with an anti-CD42c-Alexa 555 antibody (red). Nuclei were stained with DAPI (blue). Scale bar, 20 μ m. The Figure shows two representative images of samples from three individuals. Arrows denote IL-21R α ⁺ MKs. Inserts represent merged views (2.2 fold larger scale).

Figure 2. IL-21R activity changes during *in vitro* differentiation of MKs. CD34⁺ cells were cultured as described in Figure 1, scheme 1. On day 7 or 12 of culture, the cells were preincubated for 5 h in serum-free medium without cytokines, before stimulation with TPO and/or IL-21. The cells were then fixed, permeabilized and labeled with anti-CD41/CD61-ECD, -pSTAT3-Alexa 647 and -pSTAT5-Alexa 488 antibodies. Representative FC dot plots showing STAT3 and STAT5 phosphorylation in CD41/CD61⁺ cells after stimulation as indicated on days 7 and 12 of culture. Values indicate the mean \pm SEM of the percentage of events in each quadrant in three independent experiments.

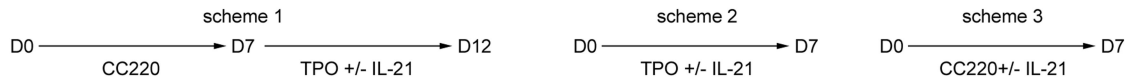
Figure 3. IL-21 promotes *in vitro* proliferation of MK progenitors through the JAK3/STAT3 pathway. CD34⁺ cells were grown in the presence (filled bars) or absence (empty bars) of IL-21 (100 ng/mL). (A) 2,500 CD34⁺ cells were plated in quadruplicate on collagen-based medium containing TPO, IL-3 and IL-6 for 10 days. *Left panels:* Representative CD41⁺ MK colony types. Scale bar, 200 μ m. *Right panels:* Numbers of MK colony types per plate, n=3 in quadruplicates. (B to D) CD34⁺ cells were cultured for 7 days in the presence of TPO, with or without IL-21 (Figure 1A, scheme 2). (B) Representative FACS plots of CD41 and CD34 distribution among 7AAD⁻ cells. (C) Total live cells were counted at day 7 and reported to the number of seeded CD34⁺ cells (n=11). (D) Numbers of CD41⁺ live cells per seeded CD34⁺ cells were deduced after FC analysis (n=11). (E) CD34⁺ cells were cultured in the presence of TPO (100 ng/ml), 100 μ g/mL polyclonal human immunoglobulins, in the absence or the presence of IL-21 (100ng/mL) and ATR-107 mAb (100 ng/mL) (n=3, in duplicates). Viable cells were counted on day 7, and results were normalized relatively to the mean number of cells obtained at day 7 in the presence of TPO alone (relative proliferation index, RPI). Then, the cells were labeled with anti-CD41 mAb and 7AAD to exclude dead cells. The percentage of CD41⁺ cells among viable cells was determined by FC. (F) CD34⁺ cells were cultured in duplicates for 7 days in the presence of different combinations of TPO (40 ng/ml), IL-6 (100 ng/mL) and soluble IL-6R (200 ng/mL) and IL-21 (100 ng/mL). On day 7, the RPI and the ratio of CD41⁺ cells among viable cells were calculated by FC (n=3, in duplicates). (G and H) CD34⁺ cells were cultured for 12 days as described in figure 1A, scheme 1. (G) IL-21 was added at the indicated concentrations and analyzed on day 12 as in C (n=6). (H) On day 7 of culture, the cells were washed, preincubated for 1 h in cytokine-free medium containing Stattic (300 nM), Tofacitinib (1500 nM) or vehicle (DMSO, 0.25%) and then cultured in the presence of TPO, with or without IL-21 (n=5). Values are reported as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; A, C-E, t-tests; F, one-way and G, two-way ANOVA followed by a Bonferonni post-hoc test.

Figure 4. Hydrodynamic gene transfer of IL-21 promotes megakaryopoiesis *in vivo*. The IL-21 expression vector (pLIVE-IL21) or control empty vector (pLIVE) were

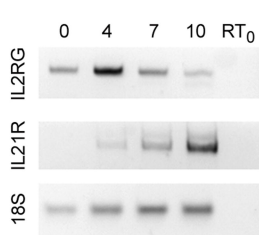
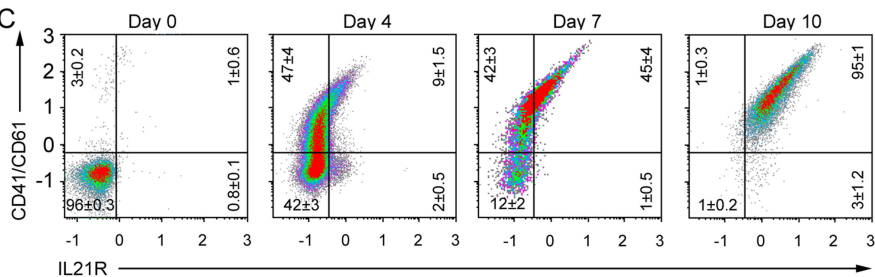
hydrodynamically transferred into mice. Six days later, the BMs and spleens were recovered and hematopoietic precursor cells were analyzed by FC. Representative dot plot analyses of bipotent erythroid-megakaryocyte progenitors (BEMP), megakaryocytic (PreMK) and erythroid (PreE) BM precursors (A) and corresponding spleen cells (B). Values are the mean \pm SEM of the percentages of gated cells among total viable cells (A, n=9, data were analyzed using a t-test; B, n=3). (C) BM cells and spleen cells of hydrodynamically transfected mice were plated in quadruplicate in a collagen-based medium in the presence of TPO, IL-6 and IL-3. MKs were identified by cytochemical staining of acetylcholinesterase activity. *Upper row:* Representative images of BM-derived pure MK colonies, mixed MK-containing colonies and non-MK colonies. *Lower panels:* Number of colonies of each type per 50,000 plated BM cells (left), and 10^6 plated spleen cells (right) (n=6). Values are the mean \pm SEM. Data were analyzed using a t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D) Femurs were harvested six days after hydrodynamic transfection. *Left panel:* Representative DAPI (blue) and CD42c staining (green) of transversal sections of femoral BM. Bar, 100 μ m. *Right panel:* Quantification of the number of MKs per mm² of BM cross section. Three 120 μ m spaced whole transversal sections per femur from six mice/group were analyzed (Data were analyzed using a t-test).

Figure 5. IL-21 promotes platelet clearance *in vivo*. The IL-21 expression vector (pLIVE-IL21) or control empty vector (pLIVE) were hydrodynamically transferred into mice. (A) On the indicated days after transfer, the total platelet count (left), the percentage of TO^{bright} platelets (middle) were measured and, the absolute count of reticulated TO^{bright} platelets was deduced (right). (B) Five days after hydrodynamic transfection, EGFP⁺ platelets were transfused and their clearance was monitored by FC. (C) Six days after hydrodynamic transfection of pLIVE-IL21 or pLIVE, the spleens (left diagrams) and livers (right diagrams) were harvested, weighed, fixed, immunostained and analyzed by immunofluorescence microscopy and CD42c⁺ platelet and F4/80⁺ macrophage areas were measured. Three sections per organ and a minimum of three micrographs per section were analyzed from three animals per group. Data were analyzed using a t-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(D) Mice were splenectomized and 7 days after the surgery were hydrodynamically transfected with pLIVE (open symbols, n=6) or pLIVE-IL-21 (grey symbols, n=7). Total (tot) and reticulated (ret) platelet counts were determined at indicated days. Differences in counts of reticulated platelet were analyzed using a t-test; * $P<0.05$, *** $P<0.0001$.

A

Culture schemes for *in vitro* differentiation of MKs from CD34⁺ progenitors

B**C****D**