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Paracrine regulation of megakaryo/thrombopoiesis by macrophages

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Objective. **Megakaryo/thrombopoiesis is a complex process regulated by multiple signals provided by the bone marrow microenvironment. Because macrophages are relevant components of the bone marrow stroma and their activation induces an upregulation of molecules that can regulate hematopoiesis, we analyzed the impact of these cells on the control of megakaryocyte development and platelet biogenesis.**

Materials and Methods. **The different stages of megakaryo/thrombopoiesis were analyzed by flow cytometry using an in vitro model of human cord blood $CD34^+$ cells stimulated with thrombopoietin in either a transwell system or conditioned media from monocyte-derived macrophages isolated from peripheral blood. Cytokines secreted from macrophages were characterized by protein array and enzyme-linked immunosorbent assay.**

Results. **Resting macrophages released soluble factors that promoted megakaryocyte growth, cell ploidy, a size increase, proplatelet production, and platelet release. Lipopolysaccharide stimulation triggered the secretion of cytokines that exerted opposite effects together with a dramatic switch of $CD34^+$ commitment to the megakaryocytic lineage toward the myeloid lineage. Neutralization of interleukin-8 released by stimulated macrophages partially reversed the inhibition of megakaryocyte growth. Activation of nuclear factor κB had a major role in the synthesis of molecules involved in the megakaryocyte inhibition mediated by lipopolysaccharide-stimulated macrophages.**

Conclusions. **Our study extends our understanding about the role of the bone marrow microenvironment in the regulation of megakaryo/thrombopoiesis by showing that soluble factors derived from macrophages positively or negatively control megakaryocyte growth, differentiation, maturation, and their ability to produce platelets.** © **2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Megakaryo/thrombopoiesis is the cellular process in which megakaryocyte progenitors progress through commitment, proliferation, and differentiation, leading to the production of platelets [1]. This process takes place within the bone marrow microenvironment and is defined by its function as a complex of cells and cell products critical for the maintenance and regulation of stem cells and their progeny. The cellular components of human bone marrow stroma include hematopoietic-derived cells, mainly macrophages, and stromal cells (endothelial cells, adipocytes, chondrocytes, and cells of the osteogenic lineage) [2]. Macrophages have long been known as critical components of both the in vivo and in vitro bone marrow milieu that regulate hematopoiesis. In vivo, resident macrophages in bone marrow play a supportive role in erythropoiesis by preventing apoptosis of the erythroid precursors, providing the iron needed for erythropoiesis, as well as phagocyting the nuclei derived from enucleated erythroblasts [3]. Almost all of the in vitro studies related to stromal macrophage effects in hematopoiesis were undertaken using long-term cultures where macrophages can form up to 40% of the total culture [4]. Macrophages are known to secrete large quantities of several cytokines, including interleukin (IL)-1, IL-6, granulocyte-colony stimulating factor, tumor necrosis factor−α (TNF-α), platelet-derived growth factor, transforming

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growth factor−β, and macrophage-colony stimulating factor, all of which can affect both stromal and hematopoietic cells [5–7]. Although several studies showed that stromal cells regulate megakaryocyte growth by the release of thrombopoietin (TPO), platelet factor 4, and chemokines such as CXCL7 and neutrophil-activating peptide (NAP-2), among others [8–10], the precise role of macrophages in the regulation of human megakaryo/thrombopoiesis is largely unknown. In this context, it has been shown that myeloid cells can regulate progenitor cell proliferation, both in a direct form [11] and in response to stromal cell stimulation [10]. Previous to TPO discovery, it was reported that monocytes synergistically cooperate with T cells in the generation of human megakaryocyte progenitor cell-derived colonies; however, high concentrations of monocytes inhibit megakaryocyte formation through prostaglandin E_2 generation [12]. In a more pathophysiological context, Alves-Rosa et al. showed, in a murine model, that macrophages regulate megakaryocyte growth and platelet numbers, suggesting that macrophages have an important regulatory role in mice megakaryopoiesis [13,14]. To further extend these findings to the human model, we used human monocyte-derived macrophages under resting conditions and stimulated with lipopolysaccharide (LPS), a product of the Gram-negative bacterial cell wall, which potently stimulates the innate immune system, eliciting both pro- and anti-inflammatory responses, as a model of macrophage activation [15]. Our results show that while resting macrophages stimulate megakaryo/thrombopoiesis, macrophage activation negatively modulates megakaryocyte growth, maturation, and platelet production.

Materials and methods

Reagents

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum were purchased from HyClone, Ficoll Hypaque, GE Healthcare (Uppsala, Sweden) and serum substitute BIT 9500 from Stem Cell Technologies (Vancouver, BC, Canada). The TPO was from Peprotech (Veracruz, Mexico) and the LPS obtained by *Escherichia coli* 0111:B4 from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti-human IL-8 neutralizing antibody and the control IgGs were from Peprotech. The nuclear factor κB (NF-κB) inhibitors, 3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 11-7082) and 6-(phenylsulfinyl)tetrazolo[1,5-b]pyridazine (Ro 106-9920) were from Biomol (Plymouth Meeting, PA, USA) [16] and Tocris (Ellisville, MO, USA) [17], respectively.

Purification of $CD34^+$ cells

Umbilical cord blood was collected after normal, full-term deliveries with the informed consent of the mother and used within 24 hours. After collection, $CD34^+$ cells were purified as described previously [18]. Briefly, the samples were diluted by one third in phosphate-buffered saline (PBS) and centrifuged to remove platelets (180*g*, 15 minutes). Low-density mononuclear cells collected from the interface of a Ficoll Hypaque gradient (480*g*, 15 minutes) were washed and resuspended in PBS containing EDTA (2 mM) and human albumin (0.5%). The $CD34^+$ cells were purified using a magnetic cell-sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). After two Mini-MACS column separations, the purity of the cell suspension was determined by flow cytometry using a FACscan flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA), and it was found that the $CD34^+$ cells typically ranged between 95% and 99%.

Culture of human monocyte-derived macrophages

Human peripheral blood monocytes were isolated from the buffy coats of healthy volunteer donors. All subjects signed written consent. The samples were first centrifuged (180*g*, 15 minutes) to remove the platelets. Then, the mononuclear cells were harvested after centrifugation (480*g*, 25 minutes) on a Ficoll-Hypaque gradient, washed, resuspended in IMDM containing 1% fetal bovine serum and plated (5×10^6 mononuclear cells/well) on 24-well dishes for 1 hour at 37°C. After removing nonadherent cells, the purity of the remaining adherent cells was measured by flow cytometry (>95% were $CD14^+$ cells). Macrophages were obtained by culturing the monocytes for 5 to 7 days in IMDM containing 5% fetal bovine serum, penicillin, and streptomycin. The differentiation of monocytes into macrophages was confirmed by flow cytometric analysis of CD71 (BD Bioscience) expression (>90% positive cells) [19]. In order to stimulate macrophages, the cells were pretreated with LPS (1 μg/mL) for 1 hour. In selected experiments, the cells were treated with two NF-κB inhibitors, BAY 11-7082 and Ro 106-9920, for 1 hour before LPS stimulation. Then, the supernatants were removed and after extensive washing the cell monolayers were incubated with fresh medium.

Coculture of $CD34^+$ and macrophages

The $CD34^+$ cells (1×10^4) were seeded in a transwell insert (0.4-mm mesh; Corning Costar, Corning, NY, USA) and cultured for 14 days in IMDM supplemented with BIT 9500 and TPO (25 ng/mL). The bottom chamber contained either no cells (control) or macrophages, obtained as described here.

Conditioned media

Resting or stimulated macrophages were incubated for 24 hours in culture medium (IMDM, BIT 9500, and antibiotics). Then, supernatants were collected, clarified by centrifugation and stored at −20°C until use. Macrophage stimulation with LPS was carried out as described here. To rule out the possibility that traces of LPS remained in the supernatants, some experiments were performed in presence of polymixin B (10 μg/mL) and the same results for megakaryocyte commitment and cell proliferation were observed (data not shown).

Megakaryocyte generation

Megakaryocyte generation was triggered by the addition of TPO (25 ng/mL) at day 0 and 7 to $CD34^+$ cells (1×10^4) placed in a transwell system or in 96-well tissue-culture plate with conditioned media. After 14 days, the absolute number of megakaryocytes was determined by multiplying the total cell number by the percentage of megakaryocytes ($CD41^+$ cells) identified by flow cytometry.

In the IL-8 neutralization experiments, conditioned media were preincubated for 15 minutes at 4°C with 10 μg/mL anti-human

IL-8 neutralizing rabbit antibody or isotypic immunoglobulins as controls.

Flow cytometry

The cells were stained with fluorescein isothiocyanate- or phycoerythrin-labeled monoclonal antibodies and evaluated for megakaryocytes (CD41), leukocyte common antigen (CD45, absent from erythrocytes and platelets), monocytes (CD14), monocytes/granulocytes (CD15), B lymphocytes (CD19), T lymphocytes (CD3), lineage-committed progenitor cells (CD38), and hematopoietic progenitors (CD34). Megakaryocyte maturity was evaluated using an anti-CD42b (GPIb) antibody. Appropriate monoclonal antibody isotypes were used as a control for each determination. All antibodies were from BD Biosciences.

Megakaryocyte ploidy

Cells were centrifuged for 10 minutes at 220g, washed, and fixed in 70% ethanol at −20°C overnight. Cells were then washed, resuspended in PBS, and incubated for 30 minutes at room temperature with saturating concentrations of fluorescein isothiocyanate−conjugated anti-CD41 or the isotype control, 1 μg/mL propidium iodide, and 100 μg/mL RNaseA. Cell ploidy in the $CD41^+$ population was analyzed by flow cytometry [20]. Mean forward scatter height of 2 N and ≥4 N megakaryocyte populations was determined as a measure of relative size [21].

Proplatelet formation

The proplatelet formation (PPF) was analyzed as previously described with some modifications [22]. Twelve-millimeter glass coverslips were coated with 100 μg/mL fibrinogen for 2 hours at room temperature and subsequently blocked with 1% bovine serum albumin for 1 hour. Cells were cultured for 13 days before being harvested, plated on coated coverslips in 24-well plates (1×10^5 cells per well) and cultured for 16 hours at 37°C and 5% CO_2. The PPF was then evaluated using fluorescence microscopy by staining the cells with tetramethyl rhodamine isothiocyanate−conjugated phalloidin. 4′,6-diamidino-2-phenylindole was used as a counterstain. Proplatelet-forming megakaryocytes were identified as large cells exhibiting long filamentous structures by fluorescence microscopy. The extent of PPF was calculated as the percentage of proplatelet-bearing cells by counting 200 cells per treatment.

Determination of the number of platelets produced in culture

The platelets were counted using flow cytometry as described previously [20]. Briefly, after 15 days of culture, the cells were incubated with a fluorescein isothiocyanate−CD41 antibody or an irrelevant isotype for 15 minutes and fixed with 1% paraformaldehyde for 20 minute. Cells from each culture condition were distributed in the same volume. For each sample, the acquisition rate was 1 μL/s for 100 s. Events were collected without gating using a log scale for size (forward scatter) and intracellular granularity (side scatter). An analytical gate was determined based on the scatter properties of normal blood platelets treated similarly to the culture-derived platelets. This gate excluded large contaminating cells and small debris or microparticles. Culture-derived platelets were counted as $CD41^+$ events with the same scatter properties as human peripheral blood platelets.

Cytokine/chemokine protein detection

The pattern of cytokines released in the supernatants of resting or stimulated macrophages was determined using a commercial human cytokine antibody array that detected 42 cytokines/chemokines simultaneously (Cytokine Array III; RayBiotech, Norcross, GA, USA). To avoid a possible high background, we diluted the samples of conditioned media by 1:5, a dilution that showed the same biological effect as with undiluted supernatants. The concentrations of IL-1β, TNF-α, monocyte chemotactic protein-1 (MCP-1), granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-8 in the culture's supernatant were determined using commercially available Ready Set and Go enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

Statistics

Results were expressed as mean ± standard error of mean of the data obtained from the separate experiments. Statistical significance was determined by one-way analysis of variance, followed by the Newman-Keuls test or a two-tailed Student's *t*-test when appropriate; p values <0.05 were considered as being statistically significant.

Results

Influence of macrophages on megakaryocyte generation from $CD34^+$ cells stimulated by TPO

We first examined the role of macrophages on megakaryocyte growth using a transwell system in which $CD34^+$ cells were cocultured over a monolayer of macrophages inside an insert that prevented cell−cell contact (see Materials and Methods). The presence of resting macrophages promoted an increase in the number of megakaryocytes (Fig. 1A), whereas macrophage activation by LPS resulted in a marked reduction of megakaryocyte generation (Fig. 1A). Although in both conditions there was an increase in total cell number, phenotypic analysis showed that while unstimulated macrophages did not modify the frequency of $CD41^+$ cells, pretreatment with LPS dramatically inhibited the commitment of $CD34^+$ cells toward the megakaryocyte lineage (Fig. 1B) by favoring the development of myeloid/granulocytic ($CD14^+$, $CD15^+$) cells (Table 1). The effects observed were not due to the presence of traces of LPS because similar results were obtained in the absence or presence of polymixin B (10 μg/mL), In addition, the stimulation of $CD34^+$ cells by TPO in the presence of LPS did not modify proliferation or commitment to the megakaryocyte lineage (Fig. 1D, E).

In order to determine whether or not the effect mediated by the macrophages was linked to the release of labile molecule(s), $CD34^+$ cells were cultured in conditioned media derived from unstimulated or LPS-activated macrophages in the presence of TPO. The results were similar to those obtained in the transwell system, suggesting that macrophages regulate megakaryocyte generation by releasing soluble and stable factor(s) (Fig. 1A). Based on these observations, for the rest of the experiments we only used conditioned media from unstimulated (MØ) or LPS-stimulated macrophages ($MØ_{LPS}$).

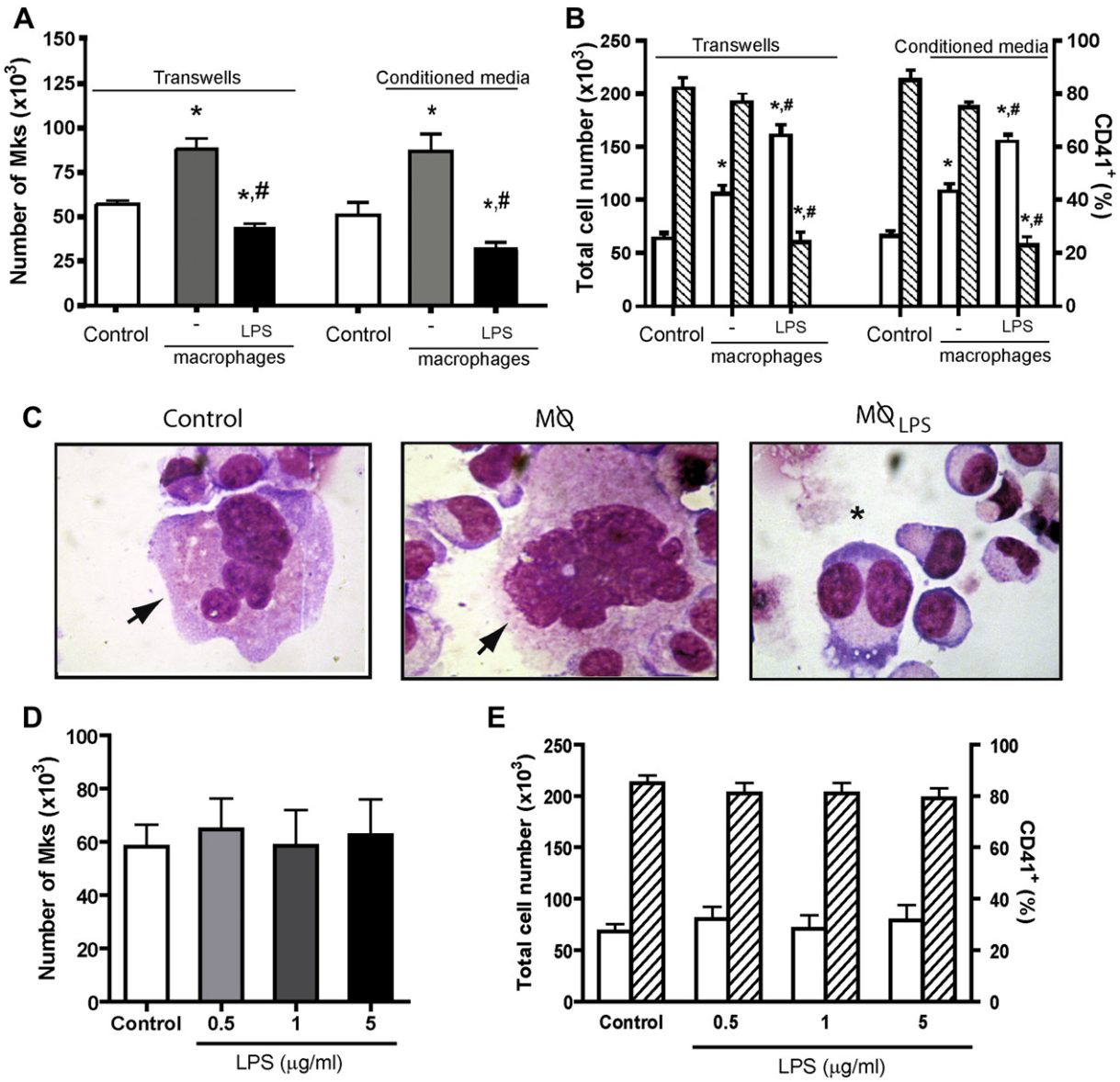

Figure 1. Effect of soluble factors secreted by macrophages on megakaryocyte generation. (**A**) Total number of megakaryocytes obtained by culturing $CD34^+$ cells (1×10^4) in a transwell system or in conditioned media from resting or LPS-stimulated macrophages. The absolute number of megakaryocytes was determined by multiplying the total cell number by the percentage of $CD41^+$ cells. (**B**) Total cell number was determined by using a Neubauer chamber (white bars) and the percentage of $CD41^+$ cells by flow cytometry (striped bars), $^*p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. macrophages without stimulus. (**C**) The cells (3.5×10^4) were cytocentrifuged and stained with May-Grünwald-Giemsa (magnification ×1000), the black arrows designate megakaryocytes with polylobulated nucleus and asterisk indicates the presence of a tetraploid megakaryocyte. (**D**) Number of megakaryocytes obtained after 14 days of culture of $CD34^+$ cells in media with TPO and different concentrations of LPS. (**E**) Total cell number (white bars) and the percentage of $CD41^+$ cells (striped bars) generated in LPS- and TPO-stimulated $CD34^+$ cells.

Regulation of megakaryocyte maturation and platelet biogenesis by macrophage-derived conditioned media

In order to analyze whether or not macrophages influence the maturation process of megakaryocytes, we examined different markers of cell maturation by flow cytometry, including the expression of GPIb (CD42b), which is expressed at more differentiated stages of megakaryocyte development, the degree of polyploidization, and the size (mean forward scatter height for each ploidy class) of the megakaryocytes. While MØ did not modify the percentage of $CD42b^+$ in the megakaryocyte population ($CD41^+$ cells), the $MØ_{LPS}$ significantly decreased the frequency of these cells (Fig. 2A). Furthermore, whereas MØ induced a moderate but statistically significant megakaryocyte polyploidization, activation of macrophages by LPS resulted in a significant decrease in $CD41^+$ cells with a DNA content ≥8 N compared to control samples (Fig. 2B). The opposing effects of MØ and $MØ_{LPS}$ were also

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Table 1. Phenotypic analysis of the progeny of TPO-stimulated $CD34^+$ cells

	CD41	CD14	CD15	CD38	CD45	CD34
Control	81 ± 2	4 ± 0.5	2 ± 1	1 ± 0.1	98 ± 1	4 ± 1
MØ	77 ± 2	15 ± 2[a]	10 ± 3[a]	2 ± 0.1	98 ± 1	4 ± 2
$MØ_{LPS}$	24 ± 2[a,b]	28 ± 4[a,b]	45 ± 2[a,b]	12 ± 3[a,b]	100 ± 1	2 ± 1

$CD34^+$ cells were cultured during 14 days in the presence or absence of conditioned media derived from macrophages supplemented with TPO. Phenotypic analysis for each population was done by flow cytometry.
[a] $p < 0.05$ vs. control.
[b] $p < 0.05$ vs. MØ.

observed in the size of the megakaryocytes at all ploidy levels (Fig. 2C).

Megakaryocyte maturation culminates with the formation of long, branching processes called proplatelets, in a process that consumes nearly the entire cytoplasmic complement of membranes, organelles, granules, and soluble macromolecules [23]. These proplatelets are then released into the bloodstream where finally they produce functional platelets [24]. Figure 2D and E shows that a higher proportion of megakaryocytes producing proplatelets together with a greater number of released platelets were obtained in the cultures of $CD34^+$ cells grown in MØ compared to the control samples. Conversely, a marked decrease in both parameters was detected in cultures grown in $MØ_{LPS}$ (Fig. 2D, E). Of note, the differences found in platelet number were not only associated with the total number of megakaryocytes, but also with the individual cell platelet production, because the amount of platelets generated by each megakaryocyte was also differentially regulated in the presence of MØ or $MØ_{LPS}$ compared to the control samples (C: 16 ± 2; MØ: 22 ± 1.7*; $MØ_{LPS}$: 4 ± 0.6*, #, $*p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. MØ, n = 4).

Cytokine profile of supernatants derived from unstimulated or LPS-activated macrophages

The type and the amount of cytokines that are produced by cells are under specific regulations. Upon activation, a vast variety of cytokines are produced, which in turn determine the type of inflammatory mediators produced by macrophages. Having demonstrated that supernatants derived from macrophages modulate megakaryocyte generation and maturation, the next question was which cytokines/growth factors were mediating these effects. Thus, we performed a cytokine antibody array analysis to characterize the cytokine profile produced in MØ and $MØ_{LPS}$. Figure 3A shows that resting macrophages released several molecules with hematopoietic activity, mainly IL-8, MCP-1, and the chemokine growth-related oncogene (GRO). Low levels of GM-CSF were also detected in some experiments; however, this cytokine showed variability between donors. The LPS-stimulated macrophages displayed a different pattern of molecules, including higher levels of GRO, GM-CSF, and MCP-1 than those observed in MØ cytokines, as well as molecules that were not observed under basal conditions, such as IL-6, IL-1β, and epithelial neutrophil-activating peptide 78 (ENA-78). Levels of IL-8 remained unchanged between treatments. In order to quantify the levels of the main cytokines known to be involved in the regulation of megakaryo/thrombopoiesis, we performed ELISA assays. In addition, because TNF-α is another macrophage-derived cytokine well known to affect megakaryocyte development [25], although it was not detected by the array, we also analyzed its levels in MØ and $MØ_{LPS}$ by ELISA. As shown in Figure 3B, resting macrophages secrete low levels of MCP-1 and GM-CSF, but not TNF-α, IL-1β, or IL-6. Macrophage stimulation with LPS significantly increased the levels of all these cytokines in the supernatants. In contrast to the results obtained in the cytokine arrays, the ELISA showed that the secretion of IL-8 was significantly upregulated upon LPS stimulation. Among the different cytokines released by the macrophages in our system, IL-8 is characterized as a strong suppressor of megakaryocyte development [26]. Therefore, we investigated whether or not the decrease in $CD41^+$ cells mediated by $MØ_{LPS}$ was a result of the marked increased in the level of this chemokine by using a neutralizing antibody. Table 2 shows that the IL-8 blockade significantly reduced the inhibition in $CD41^+$ frequency observed in $CD34^+$ cultures grown in conditioned medium from $MØ_{LPS}$.

NF-κB activation is required for the inhibitory activity on megakaryocyte generation mediated by $MØ_{LPS}$

The activation of macrophages may involve several transduction pathways depending on the agonist implicated. To determine whether the activation of NF-κB, the canonical pathway of LPS [27,28], was responsible for the inhibition of megakaryocyte generation mediated by $MØ_{LPS}$, we examined the effect of two specific inhibitors of NF-κB, i.e., BAY 11-7082 and Ro 106-9920 (both at 10 μM, a concentration that did not alter cell survival) [17,29,30]. Figure 4A shows that while $CD34^+$ cells grown with TPO in the presence of conditioned media from $MØ_{LPS}$ dramatically reduced the number of megakaryocytes in culture, $MØ_{LPS}$ that were pretreated with the NF-κB inhibitors generated significantly more megakaryocytes than the control samples. This effect was associated with a complete disappearance of the inhibitory action on $CD34^+$ cell commitment toward the megakaryocyte lineage without modifying cell proliferation (Fig. 4B).

Discussion

Our study presents evidence to support the theory that soluble factors released from macrophages have the potential to regulate not only megakaryocyte development but also platelet biogenesis. According to the activation status, macrophages positively or negatively regulate megakaryo/thrombopoiesis.

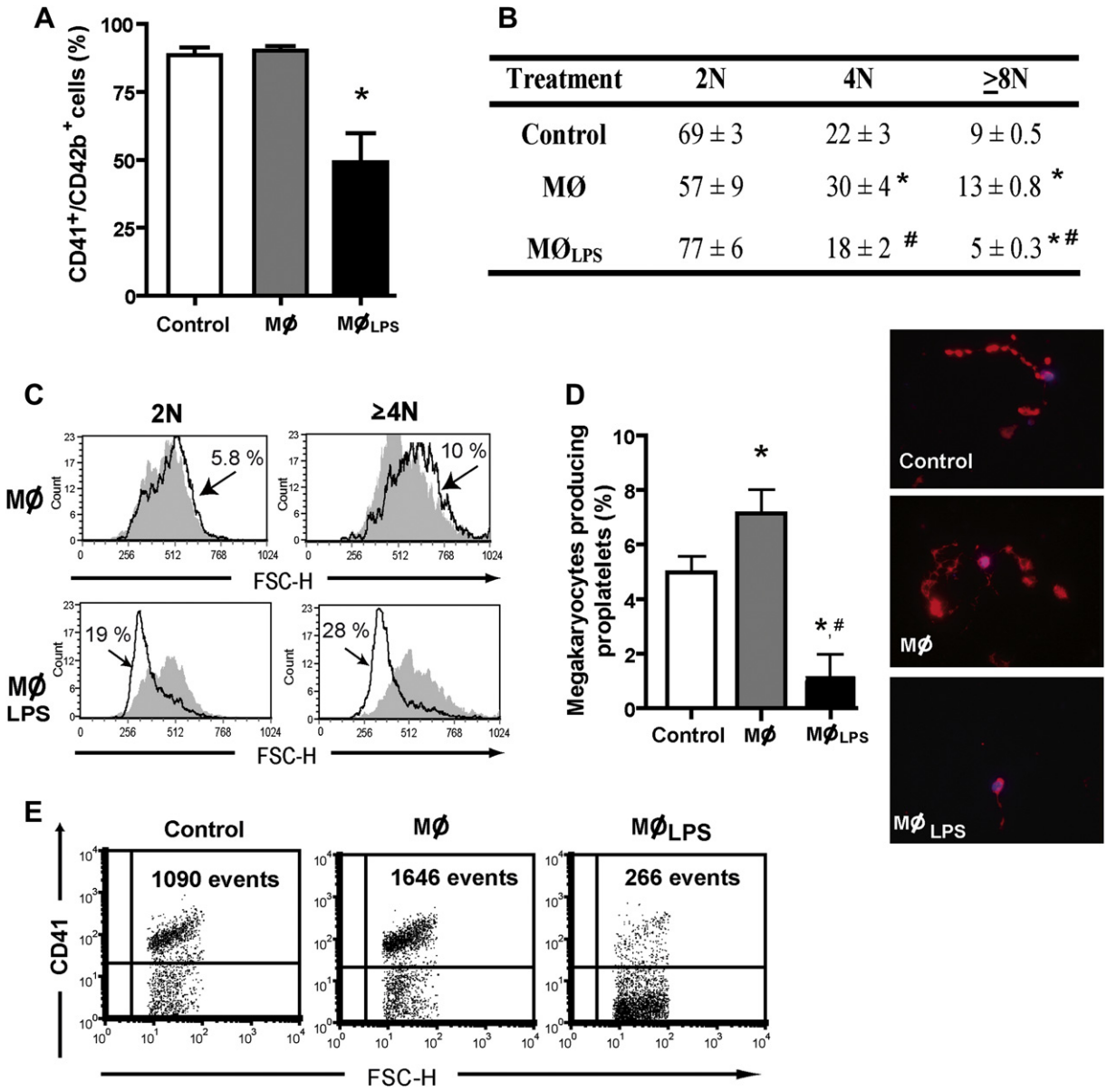

Figure 2. Macrophages regulate the maturation of megakaryocytes and platelet biogenesis. $CD34^+$ cells were cultured with conditioned media from resting or LPS-stimulated macrophages for 14 days. (**A**) CD42b expression on megakaryocytic cells ($CD41^+$ cells). (**B**) Polyploidy levels on $CD41^+$ cells were evaluated by propidium iodide staining (n = 5). (**C**) Cell size (mean forward scatter height) of megakaryocytes cultured in growth media (gray histogram) or conditioned media (black histogram) was measured for each ploidy level by flow cytometry. The numbers at right indicate the percentage of size increase, and the numbers at left the percentage of size diminution. The figure represents one of four independent experiments. (**D**) Proplatelet-bearing megakaryocytes were counted by fluorescence microscopy. Values show the mean ± standard error of mean of four independent experiments. (**E**) Representative flow cytometry analysis showing culture-derived platelets (n = 4). $*p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. MØ.

Resting macrophages promote megakaryocyte proliferation, maturation, polyploidization, and their capacity to produce proplatelets and release platelets. The observation that macrophages increase megakaryocyte numbers is consistent with the known role of these cells in the regulation of hematopoiesis. Previous studies focused on the role of macrophages in the regulation of erythropoiesis. That macrophages have a main role in erythropoiesis is not only supported by the fact that these cells form erythroblastic islands within bone marrow, but also because they provide both negative and positive signals for the growth of erythroid progenitor cells [31]. More recently, macrophages have emerged as potential regulators for all hematopoietic lineages, since a subpopulation of macrophages that support osteoblast function are pivotal for maintaining the endosteal hematopoietic stem cell niche [32]. Indirect evidence that monocytes/macrophages positively regulate megakaryocyte growth was also shown by earlier studies in which it was

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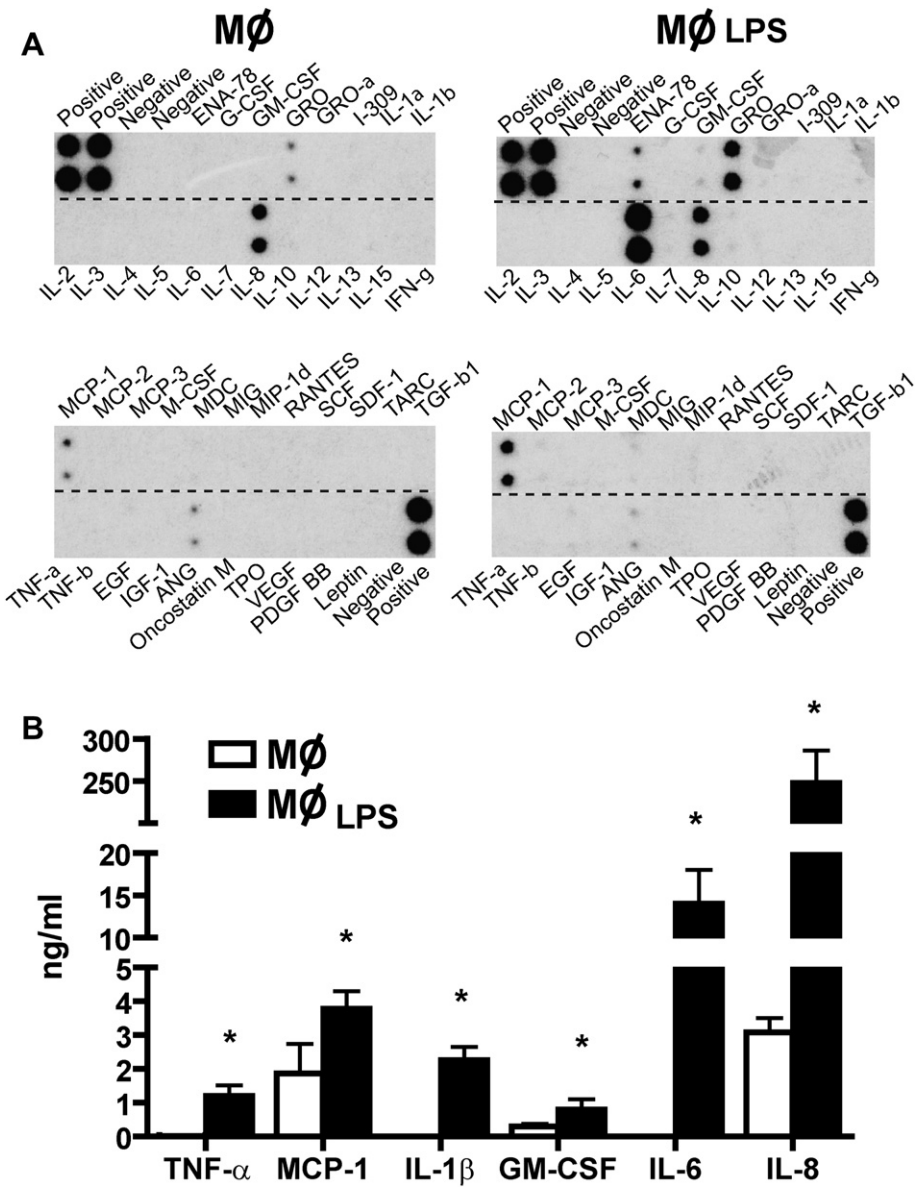

Figure 3. Measurement of soluble factors released by macrophages. Macrophages were incubated in serum-free medium for 24 hours and supernatants of unstimulated or LPS-stimulated macrophages were collected. (**A**) The antibody array was incubated with supernatants from resting or LPS-stimulated macrophages (n = 3 from three different donors). The figure shows duplicate spots for each cytokine/growth factor and represents one experiment. G-CSF = granulocyte-colony stimulating factor; IFN = interferon; M-CSF = macrophage-colony stimulating factor; MDC = macrophage-derived chemokine; MIG = monokine induced by IFN-γ; MIP = macrophage inflammatory protein; RANTES = regulated upon activation normal T-cell expressed and secreted; SCF = stem cell factor; SDF-1 = stromal cell derived factor-1; TARC = thymus and activation regulated chemokine; TGF-β1 = transforming growth factor-β1 (only the active form of this cytokine is detected by this array); EGF = epithelial growth factor; IGF = insulin-like growth factor; VEGF = vascular endothelial growth factor; PDGF = platelet-derived growth factor. (**B**) TNF-α, MCP-1, IL-1β, GM-CSF, IL-6, and IL-8 concentrations in conditioned media were measured by ELISA (n = 5). *$p < 0.05$ vs. MØ.

demonstrated that bone marrow stroma, of which >40% is comprised of macrophages, synergized with TPO to stimulate megakaryocyte production [33–35].

During their development, megakaryocytes undergo a series of maturation processes that include polyploidization, cytoplasmic maturation, and, finally, the formation of proplatelets and the release of platelets into blood circulation [36]. The molecular and humoral mechanisms that regulate megakaryocyte polyploidization have still not been completely identified. In agreement with the data of Pastos et al. [35], who used conditioned media from bone marrow stroma, we now show that conditioned media derived from macrophages increased the ploidy values of megakaryocytes. Moreover, consistent with these findings, we also found that the number of megakaryocytes, PPF, and platelets were higher in the presence of

Table 2. IL-8 neutralization partially restored the inhibition on megakaryocyte commitment

Percentage of $CD41^+$ cells								
Control			MØ			$MØ_{LPS}$		
None	α-IgG	α−IL-8	None	α-IgG	α−IL-8	None	α-IgG	α−IL-8
87 ± 4	83 ± 1	84 ± 3	84 ± 5	81 ± 2	83 ± 4	25 ± 4	22 ± 4	38 ± 7[a]

[a] $p < 0.05$ vs. α-IgG for the same treatment.

a macrophage-conditioned medium. By protein array and ELISA, we found that unstimulated macrophages release several cytokines/chemokines, including IL-8, MCP-1, and GRO, and low levels of GM-CSF. Although the first three chemokines are inhibitors of hematopoiesis and, in particular, IL-8 is a well-known inhibitor of megakaryopoiesis [37,38], their effects appear to be counterbalanced by the synergistic proliferative activity of GM-CSF and TPO. In fact, it has been shown that GM-CSF can synergize with TPO to increase hematopoietic progenitors [39].

Because none of the detected molecules are known to regulate megakaryocyte ploidy or platelet production, it seems reasonable to consider that the molecule(s) involved in these effects were either secreted in very small amounts that were too low to be detected by the array, or not detected by this assay. More experiments are required to identify these potential factor(s).

In contrast to resting macrophages, activation of cells by LPS decreased megakaryocyte ploidy, size, and platelet release. Notably, although there was an increase in cell proliferation, there was a decrease in megakaryocyte numbers due to a marked reduction in the frequency of $CD41^+$, which suggested an inhibitory effect in the process of megakaryocyte differentiation. A negative regulation of megakaryopoiesis by activated macrophages was also suggested by Pillai et al., who demonstrated that stromal-stimulated monocytes express and release CXCL7 peptides including NAP-2, which inhibits megakaryocyte growth [10].

LPS-stimulated macrophages upregulated the levels of most of the molecules expressed by resting macrophages and, in addition, induced the release of other molecules capable of modulating hematopoiesis, such as IL-6, IL-1β, TNF-α, GM-CSF, and ENA-78. Despite the fact that some of these inhibit the proliferation of hematopoietic progenitors whereas others have the opposite effect [11,40], the biological effects of these cytokines are context-dependent, a situation that has tended to generate apparently contradictory reports on their activity [26]. The biological effect of LPS-stimulated macrophage-conditioned media was an increase in cell proliferation together with a profound decrease in $CD34^+$ cell commitment toward the megakaryocytic lineage. Thus, a combined effect of the known proliferative activities of IL-1, IL-6, and GM-CSF most probably accounted for the marked increase in cell growth. Among the different molecules released by macrophages, IL-8 exerts an inhibitory effect on hematopoiesis and especially on megakaryopoiesis, including not only the inhibition of megakaryocyte proliferation but also differentiation [26,38,41]. Although it was secreted by resting macrophages, the levels of this chemokine were almost 100-fold higher in supernatants from stimulated macrophages, and neutralization of its activity partially restored the frequency

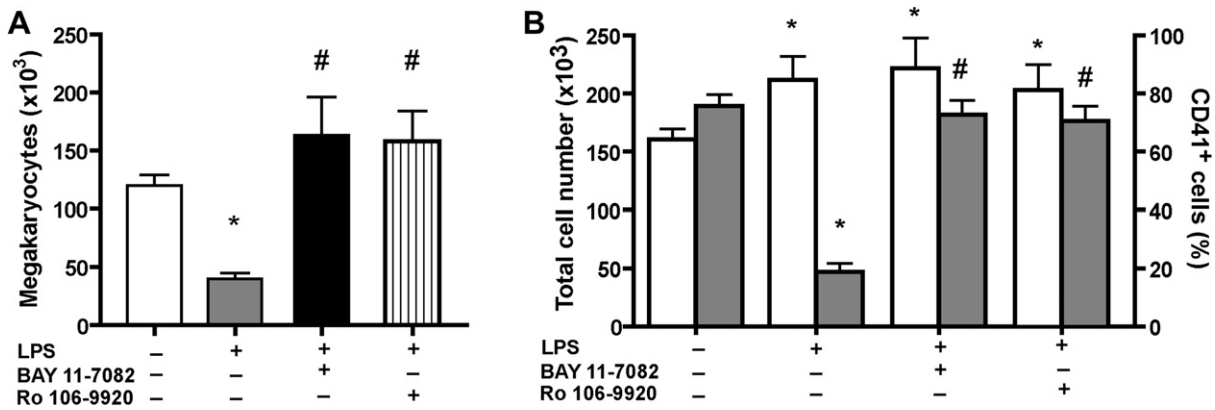

Figure 4. NF-κB is involved in the inhibition of megakaryocyte commitment mediated by LPS-stimulated macrophages. Macrophages were treated or not with BAY 11-7082 (10 μM) or Ro 106-9920 (10 μM) for 1 hour before LPS addition. The supernatants were collected and $CD34^+$ cells were cultured as described in the Materials and Methods. (**A**) Total number of megakaryocytes. (**B**) Cell proliferation (white bars) and percentage of $CD41^+$ cells (gray bars) for each treatment. *$p < 0.05$ vs. none, # $p < 0.05$ vs. LPS alone.

of $CD41^+$ cells grown in conditioned media derived from LPS-stimulated macrophages. In the same line of evidence, the levels of IL-8 were found to be significantly increased in the serum of patients with myeloid metaplasia with myelofibrosis, and neutralization of IL-8 and its receptors increased the proliferation and differentiation of these patient's $CD41^+$ cells and restored their polyploidization [38]. Interestingly, the two receptors for IL-8, CXCR1 and CXCR2, also bind other chemokines that belong to the CXC family, including NAP-2, ENA-78, and GRO [42]. Although the effect of ENA-78 and GRO on megakaryopoiesis has not yet been examined, NAP-2 is well characterized as a negative regulator of megakaryocyte generation [10,26]. Because high levels of GRO and ENA-78 were detected in the supernatants of the LPS-stimulated macrophages, it could be hypothesized that both cytokines, together with IL-8, are responsible for the impaired maturation of megakaryocytes observed in cells grown in the presence of conditioned media from LPS-stimulated macrophages. Moreover, the expression of the CXC chemokines IL-8, GRO, and ENA-78 was mediated by NF-κB [43–45], and macrophage activation by LPS results in rapid and transient NF-κB activation [28]. Here, we showed that although the increase in cell proliferation was not abolished by two different NF-κB inhibitors (BAY 11-7082 and Ro 106-9920), the inhibition of megakaryocyte commitment mediated by LPS-stimulated macrophages was entirely dependent on NF-κB. These findings indicate that different signaling pathways are involved in the synthesis of the factors modulating megakaryocyte development and strengthen the notion that CXC chemokines control the megakaryocyte differentiation process. Although IL-6 is also known as a megakaryocyte growth and maturation factor [46], and although its levels were significantly increased in the supernatant of LPS-stimulated macrophages, the maturation activity of IL-6 appears to be counteracted by that of the inhibitory chemokines.

In conclusion, we have described a direct regulatory function of macrophages in the process of megakaryo/thrombopoiesis. In resting cells, the balance of secreted molecules favors the activity of factors that promote the proliferation and maturation of megakaryocyte progenitors, whereas the opposite effect predominates in activated macrophages.

Understanding the relevant role of macrophages as a component of the bone marrow microenvironment that regulates megakaryocyte and platelet development should provide opportunities for development of new strategies for treating hematopoiesis-associated pathologies.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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