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Toll-Like Receptor-3-Activated Human Mesenchymal Stromal Cells Significantly Prolong the Survival and Function of Neutrophils

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ABSTRACT

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) are stromal precursors endowed with extensive immunomodulative properties. In this study, we aimed to assess whether Toll-like receptor-3 (TLR3)- and TLR4-activated BM-MSC influence human neutrophil (PMN) responses under coculture conditions. We show that TLR3 triggering by polyinosinic:polycytidylic acid dramatically amplifies, in a more significant manner than TLR4 triggering by lipopolysaccharide, the antiapoptotic effects that resting BM-MSC constitutively exert on PMN under coculture conditions, preserving a significant fraction of viable and functional PMN up to 72 hours. In addition, TLR3- and TLR4-activated BM-MSC enhance respiratory burst ability and CD11b expression by PMN. The coculture in the absence of cell contact and the incubation of PMN in supernatants harvested from TLR3- and TLR4-activated BM-MSC yield comparable results in terms of increased survival and immunophenotypic changes, thus suggesting the involvement of endogenous soluble factors. Neutralizing experiments reveal that the biological effects exerted on PMN by TLR3-activated BM-MSC are mediated by the combined action of interleukin 6, interferon-β (IFN-β), and granulocyte macrophage colony-stimulating factor (GM-CSF), while those exerted by TLR4-activated BM-MSC mostly depend on GM-CSF. MSC isolated from thymus, spleen, and subcutaneous adipose tissue behaves similarly. Finally, the effects exerted by TLR3- or TLR4-stimulated BM-MSC on PMN are conserved even after the previous priming of BM-MSC with IFN-γ and tumor necrosis factor-α. Our data highlight a novel mechanism by which MSC sustain and amplify the functions of PMN in response to TLR3- and TLR4-triggering and may consequently contribute to inflammatory disorders. STEM CELLS *2011;29:1001–1011*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are the precursors of tissue stromal cells and fundamental elements of tissue homeostasis [1, 2]. Originally isolated from the bone marrow (BM), where they support hematopoiesis, MSCs can be expanded from virtually all tissues [3], thus forming a complex stromal system throughout the body. Among other functions, MSC also display powerful immune modulatory properties toward the main immune effector cells [4–6]. The activation of T lymphocytes by either TCR-dependent [7] or unspecific stimuli [8] is prevented by BM-derived MSCs (BM-MSCs) under in vitro coculture conditions; such effects are mediated by redundant mechanisms depending on both contact-dependent interactions [7] and soluble factors [6, 7]. MSCs also positively or negatively affect B cell proliferation according to their priming by inflammatory cytokines [9], and they may inhibit the reactivity of natural killer (NK) cell [4, 10] and dendritic cell toward allogeneic cells [4, 11]. Overall, MSCs and their progeny [12] seem to act as a competitive system that prevents excessive reactions toward pathogens, thus contributing to the resolution of immune responses [5–6]. Immune modulatory properties of MSC have also been demonstrated in vivo [13, 14], where they appear to be finely tuned by the local microenvironment [5, 6]. Inflammatory cytokines, such as IFN-γ or tumor necrosis factor-α (TNF-α), "prime" MSC for enhanced suppressive mechanisms [15], while infectious agents may hamper inhibitory effects of MSC through the engagement of Toll-like receptors (TLRs), such as TLR3 and TLR4 [16]. Actually, nearly opposite effects on MSC have been recently described in lymphocyte-MSC cocultures after short-term, low-level stimulation of either TLR3 or TLR4 [17]. TLR4-primed MSC exhibited a proinflammatory profile,

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with increased levels of cytokines such as interleukin 6 (IL-6), IL-8/CXCL8, or transforming growth factor-β (TGF-β), while TLR3-primed MSC exerted increased immunosuppressive activities, by producing mainly IL-10, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E_2 (PGE2) [17]. In analogy with the M1/M2 monocyte/macrophage polarization [18], Waterman et al. [17] hypothesized a similar MSC functional polarization into MSC1 (proinflammatory) versus MSC2 (immunosuppressive) phenotype.

Neutrophils (PMN) are phagocytic cells of the innate immune system that act as the first line of defense against infectious pathogens in the inflammatory response [19]. Upon challenge by various stimuli, PMN release lytic enzymes with powerful antimicrobial potential and generate reactive oxygen intermediates that are essential for pathogen killing [19]. PMN can also be induced to produce de novo a variety of mediators involved in their functions [20] and, in turn, cross-talk with immune [21–23] and nonimmune effector cells [24]. Although PMN are normally short-living cells, when cocultured with untreated BM-MSC for up to 40 hours they display a significantly lower tendency to undergo apoptosis, particularly if coincubated at high PMN:BM-MSC ratio (50:1) [25]. Concomitantly, the capacity of PMN in producing hydrogen peroxide upon formyl-methionyl-leucyl phenylalanine (fMLF) stimulation decreases under coculture conditions, while their chemotactic ability or their CD11b or CD62L expression remains unaffected [25]. All these effects are not only reproduced by culturing PMN in BM-MSC-conditioned supernatants but also fully dependent on BM-MSC-derived IL-6 [25, 26]. More recently, supernatants from parotid-derived MSC stimulated via TLR4 by lipopolysaccharide (LPS) also proved to significantly improve PMN survival and chemotaxis [27]; however, even if MSC supernatants were shown in this study to contain granulocyte colony-stimulating factor (G-CSF), IL-6, IL-8/CXCL8, and macrophage migration inhibitory factor, no functional analysis was performed to identify the factors specifically responsible for their effects [27].

In this study, we examined whether MSC, activated by either polyinosinic:polycytidylic acid [poly(I:C)], a specific ligand for TLR3, or LPS, a ligand for TLR4, modify PMN survival, phenotype, and function. We demonstrate here that TLR3-activated BM-MSCs are more powerful than TLR4-stimulated BM-MSC in preserving PMN viability and function, and that such effects are also shared by MSC isolated and expanded from different sources (thymus, spleen, and adipose tissue). We also show that a concerted action of endogenously produced IL-6, IFN-β, and granulocyte macrophage colony-stimulating factor (GM-CSF) determines most of the modulatory effects exerted on PMN by TLR3-activated BM-MSC, while GM-CSF is solely responsible for most of those exerted by TLR4-activated BM-MSC. These observations highlight a novel mechanism by which tissue-resident MSC, upon TLR activation, may sustain and amplify the functions of PMN.

Materials And Methods

Cell Purification and Cocultures

MSCs were isolated from BM, thymus, spleen, and subcutaneous lipoaspirates obtained from healthy donors after their informed consent and expanded in vitro as previously described [28, 29]. Following a brief expansion in Dulbecco's modified Eagle medium supplemented with 18% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all by GIBCO/Invitrogen, S.Giuliano Milanese, Italy), cells displayed a homogeneous mesenchymal immunophenotype starting from passages 2–3 (P2-3) and proved capable of in vitro multilineage differentiation into adipocytes, osteoblasts, and chondrocytes after exposure to specific differentiating media, as described elsewhere [28, 29]. PMN from buffy coats of normal volunteers were isolated under endotoxin-free conditions, as previously described [22, 30]. PMN (99.1% $\pm$ 0.5% purity) and MSC were cocultured for up to 44 hours in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (all by GIBCO/Invitrogen), at PMN:MSC ratios ranging from 1,000:1 to 10:1, in the presence or absence of 100 μg/ml of poly(I:C) (InvivoGen, San Diego, CA) or 100 ng/ml of LPS (Ultra-Pure E.coli LPS; Alexis Biochemicals, San Diego, CA), according to preliminary experiments performed to determine the optimal working concentrations (Supporting Information Fig. 1). In all cases, MSCs were plated 24 hours before the start of cocultures. In selected experiments, PMN were cultured on top of 0.4 μm pore size Transwell inserts (Corning Costar, Cambridge, MA) to prevent cell contact between PMN and MSC. In selected experiments, BM-MSCs were exposed to 200 IU/ml of recombinant human interferon γ (R&D Systems, Minneapolis, MN, Cat. # 285-IF) and 1650 IU/ml of TNF-α (R&D Systems, Cat. # 210-TA) for 48 h, according to a protocol in use in our laboratory and developed to achieve maximal immune regulatory activity (M.K., unpublished data). Thereafter, the supernatant was collected, BM-MSCs were gently washed twice with fresh medium to remove any residual cytokine, and finally cocultured with freshly isolated PMN, as described above. Under all conditions, PMN were harvested by careful pipetting at the end of the incubation, centrifuged at 600*g* for 5 minutes, and finally resuspended in phosphate-buffered saline for subsequent assays. Full integrity of MSC layers was checked in all cases. MSC-conditioned media were obtained by incubating the same MSC batches used for the cocultures with PMN in the presence or absence of poly(I:C) or LPS for 24 hours, and then by collecting and processing their cell-free supernatants. Cytokine blocking experiments were conducted by culturing PMN in MSC-conditioned media previously preincubated for 30 minutes at 37°C in the presence of specific neutralizing monoclonal antibodies (mAbs) towards G-CSF, GM-CSF (both from R&D Systems), IL-8/CXCL8 (PeproTech, Rocky Hill, NJ), IFN-γ (clone B133.3, kindly provided by Dr. G. Trinchieri, National Cancer Institute, Frederick, MD), and TNF-α (clone B154.2, kindly provided by Dr Giorgio Trinchieri, National Cancer Institute, Frederick, MD). Alternatively, PMN were preincubated for 30 minutes with anti-IL-6R (Bender MedSystems, Wien, Austria), anti-type-I IFN-R (MMHAR-2; PBL InterferonSource, Piscataway, NJ), or, in the case of the subsequent culture in LPS-conditioned supernatant, also anti-TLR4 mAbs (kindly provided by Dr Greg Elson, Novimmune, Geneve, Switzerland), before their further culture.

Cytofluorimetric Analysis

After isolation from MSC, PMN were identified on the basis of their typical morphological parameters (forward scatter/side scatter). The levels of PMN apoptosis were quantified by the Annexin-V-FLUOS staining kit (Roche Diagnostics, Mannheim, Germany) and flow cytometry analysis (FACS, BD FACScalibur). Expression of surface antigens was also assessed by FACS with fluorochrome-conjugated anti-CD16, anti-CD11b, and anti-CD64 (all from BD Biosciences, Buccinasco, Italy).

Enzyme-Linked Immunosorbent Assay

Cytokine concentrations in BM-MSC-free supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits for: human IL-6 (detection limit 0.6 pg/ml; Immunotools, Friesoythe, Germany), IL-8/CXCL8 (detection limit 1 pg/ml; Immunotools), IFN-α (detection limit 12.5 U/ml; PBL InterferonSource, Piscataway, NJ), IFN-β (detection limit 2.5 U/ml; Biosource/Invitrogen, S.Giuliano Milanese, Italy), IFN-γ (detection limit 2.0 pg/ml; clone B133.3; Immunotools), G-CSF (detection limit 0.4 pg/ml; R&D Systems), GM-CSF (detection limit 2 pg/ml; BioLegend, San Diego, CA), and TNF-α (detection limit 0.09 pg/ml; human high-sensitivity kit by Invitrogen, Cat.# KHC3014), according to the respective manufacturer's instructions.

Respiratory Burst

Superoxide anion (O_2^-) release was estimated by the cytochrome C reduction assay, as previously described [31].

Statistical Analysis

One-way analysis of variance and Holm-Sidak test for multiple comparisons were used to statistically evaluate the difference of sample means among multiple groups. A p value $<$ .05 was considered as significant. Data are represented using mean $\pm$ SD in all cases. Calculations have been performed using STATA IC v.10.0 (StataCorp, College Station, TX).

All other procedures, performed according to standard methodologies and described in previous publications by our groups, are available in full details in the Supporting Information section.

Results

BM-MSC Exert a Powerful Prosurvival Effect on PMN upon Activation by TLR3 or TLR4 Agonists

Highly purified populations of peripheral PMN were plated with in vitro expanded BM-MSC at coculture ratios ranging from 1,000:1 to 10:1, either in direct contact or in Transwell conditions. Viability and expression levels of surface markers in PMN were investigated after 20 hours of incubation, unless differently specified. As shown in Figure 1A and Supporting Information Figure 2, PMN survival was enhanced by untreated BM-MSC only at 10:1 PMN:BM-MSC ratio, going from 18.5% $\pm$ 6.6% viability in the absence of BM-MSC to 44.4 $\pm$ 9.5% (p $<$ .001) (Fig. 1A). Under the latter conditions, the protective effect of BM-MSC was already significant after 6 hours and lasted up to 44 hours of culture, although not significantly anymore (Fig. 2). Cocultures performed by using Transwell inserts yielded similar results, with PMN survival ranging from 22.9% $\pm$ 12.3% (in the absence of BM-MSC, data not shown) to 39.6% $\pm$ 10.9% ($p < .01$) (Fig. 1A and Supporting Information Fig. 2). These results substantially confirm and extend previous observations aimed at defining whether resting BM-MSC could influence PMN viability [25].

To subsequently investigate whether activated BM-MSC could exert enhanced modulatory effects on PMN survival, we performed the cocultures in the presence of poly(I:C), the specific ligand of TLR3, which is expressed and functional in BM-MSC [16, 32, 33] but not in PMN [34, 35] (Fig. 1A and Supporting Information Fig. 2). Under these conditions, the survival rate of PMN was strongly increased and already significant at the 100:1 PMN:BM-MSC ratio (49.5% $\pm$ 13.2%, p $<$ .001) but was maximum at the 10:1 ratio (73.6% $\pm$ 6.8%, p $<$ .001) (Fig. 1A and Supporting Information Fig. 2). Notably, at the 10:1 ratio, the degree of PMN survival was maintained at very high levels also at 44 hours (Fig. 2) and up to 72 hours. At the latter time point, approximately 50% of PMN were still viable (data not shown). Comparable survival levels were detected also under Transwell conditions at 44 hours (60.2% $\pm$ 12.4%, p $<$ .001) (Fig. 1A and Supporting Information Fig. 2), thus suggesting the involvement of endogenous soluble factors.

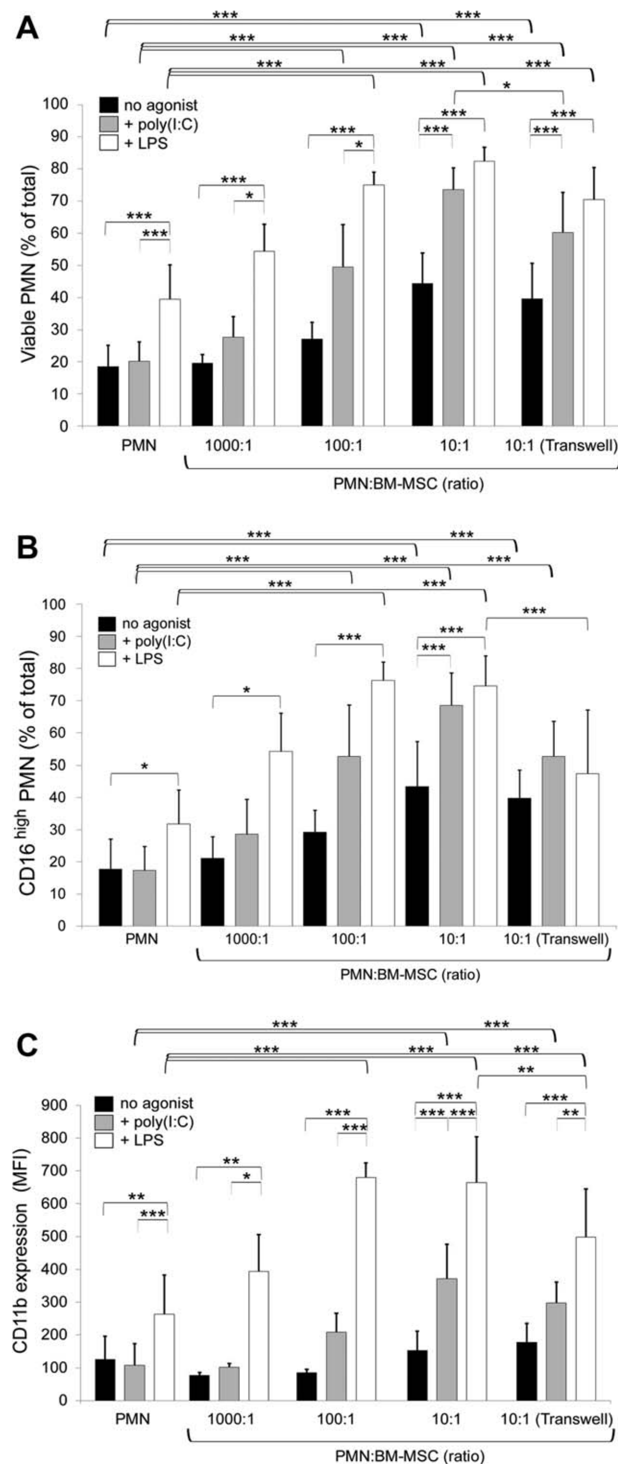
For comparison, PMN:BM-MSC cocultures were also performed in the presence of LPS, well considering, however, that both BM-MSC [16, 27, 32, 33] and PMN [30, 34] express functional TLR4, the specific LPS receptor [36], and do respond to its ligands. In fact, LPS itself, in the absence of BM-MSC, significantly delayed PMN apoptosis (Fig. 1A and Supporting Information Fig. 2), in line with the literature [19]. Nonetheless, we further observed that PMN survival was additionally increased in LPS-treated PMN:BM-MSC cocultures, being apparently more pronounced than under poly(I:C) treatment (75.0% $\pm$ 4.1%, p $<$ .001 at the 100:1 ratio; 82.4% $\pm$ 4.4%, p $<$ .001, at the 10:1 ratio) (Fig. 1A and Supporting Information Fig. 2). However, LPS activates PMN also directly and this effect has to be distinguished from that dependent only on TLR4 triggering on BM-MSC. Thus, poly(I:C)-stimulated BM-MSC was eventually more efficient than LPS-stimulated BM-MSC in protecting PMN viability, when considering the net protective effects over the basal viability of PMN cultured without TLR agonists (Supporting Information Fig. 3A), as well as the percentage of increased PMN survival observed in PMN:BM-MSC cocultures as compared to PMN-only cultures, with or without the corresponding TLR agonists (Supporting Information Fig. 3B). Accordingly, the net antiapoptotic effect of LPS was less sustained than poly(I:C) also in time-course studies, as it remarkably declined between 20 and 44 hours (Fig. 2). Significant levels of viable PMN (approximately 45%) were maintained up to 72 hours (data not shown). On the other hand, Transwell experiments proved that soluble factors were greatly responsible for the protection of PMN survival also in the case of LPS-treated PMN:BM-MSC cocultures (Fig. 1A and Supporting Information Fig. 2). Taken together, these data show that BM-MSC activated with agonists for TLR3 and—less efficaciously—TLR4 delay PMN apoptosis much more significantly than resting BM-MSC.

Immunophenotypic Changes by PMN After Coculture with Resting or TLR-Activated BM-MSC

As previously described, CD16 (FcγR-III) can be reliably used as a surrogate marker of PMN viability [37]; accordingly, we observed that the percentage of PMN retaining high levels of CD16 expression (CD16^{high} PMN) matched the percentage of viable PMN under all coculture conditions (Fig. 1B and Supporting Information Fig. 4). On the other hand, CD11b is typically modified by PMN activation [22, 31] and was used in our study as a marker of the activation status of PMN. As expected, neither the percentage of CD16^{high} PMN (Fig. 1B and Supporting Information Fig. 4) nor the expression of CD11b (Fig. 1C and Supporting Information Fig. 5) changed when PMN were cultured alone in the presence of poly(I:C). By contrast, the percentage of CD16^{high} PMN was significantly higher when PMN were cultured in the presence of BM-MSC (43.37% $\pm$ 13.92%) rather than in its absence (17.74% $\pm$ 9.38%; p $<$ .001), at the 10:1 coculture ratio (Fig. 1B and Supporting Information Fig. 4). A significant increase of CD16^{high} PMN was observed by adding either poly(I:C) (68.54% $\pm$ 10.09%; p $<$ .001) or

LPS (74.56% ± 9.43%; $p < .001$) to PMN:BM-MSC cocultures, similar to what was observed regarding the prosurvival effect. These data were comparable to the results obtained under Transwell conditions (Fig. 1B and Supporting Information Fig. 4).

On the other hand, the expression level of CD11b was unchanged by coculturing PMN with untreated BM-MSC, regardless of coculture ratios and the use of Transwell inserts (Fig. 1C and Supporting Information Fig. 5). By contrast, CD11b was significantly upregulated if poly(I:C) was added to PMN:BM-MSC cocultures (Fig. 1C and Supporting Information Fig. 5). Similarly, the direct upregulatory effects of LPS on the percentage of CD16^{high} PMN and CD11b expression levels in PMN cultured without BM-MSC were also greatly amplified by BM-MSC (Fig. 1B and 1C, Supporting Information Figs. 4, 5), peaking already at the 100:1 PMN:BM-MSC ratio. These effects appeared effectively mediated by LPS-activated BM-MSC when considering the results according to the same procedures detailed for Supporting Information Figure 3, aimed to detect the net effects of the cocultures as compared to the effect of LPS itself on PMN cultured alone (Supporting Information Figs. 6, 7). Interestingly, similar results concerning the percentage of CD16^{high} PMN and CD11b expression level were obtained under Transwell conditions in LPS-activated PMN:BM-MSC cocultures (Fig. 1B, 1C and Supporting Information Figs. 4, 5). However, the higher variability observed in cocultures using Transwell inserts partially limited the statistical significance of the immunophenotypic changes observed. Taken together, these data demonstrate that TLR3- and TLR4-stimulated BM-MSC better preserve PMN expressing high-intensity CD16 and CD11b than resting BM-MSC.



Coculture with BM-MSC "Primes" PMN for Increased Respiratory Burst Ability

The coculture with BM-MSC also modified the capacity of PMN of producing superoxide anion (O_2^-) in response to fMLF (Fig. 3A). Accordingly, while PMN cultured for 20 hours in the absence of BM-MSC and then stimulated with fMLF for up to 40 minutes was unable to release O_2^- (Fig. 3A), they properly responded to fMLF challenge if preincubated for 20 hours with LPS, but not with poly(I:C) (Fig. 3A). Remarkably, PMN previously cocultured with resting BM-MSC also displayed a significant ability to release O_2^- following exposure to fMLF, which was further enhanced if PMN:BM-MSC cocultures were carried out in the presence of either poy(I:C) or, at higher levels, LPS (Fig. 3A). The latter phenomenon is presumably due to a genuine "priming" effect exerted by poly(I:C)- or LPS-activated BM-MSC, because a comparable number of surviving PMN were detected under the two experimental conditions (Fig. 1A). Interestingly, a similar trend was observed in experiments performed after 72 hours of PMN:BM-MSC cocultures (Fig. 3B).

Figure 1. Bone marrow-derived mesenchymal stromal cells (BM-MSCs) exert a powerful prosurvival effect, maintain higher levels of CD16 expression, and strongly upregulate neutrophil CD11b expression under coculture conditions in the presence of either poly(I:C) or LPS. Neutrophils (PMN) were cultured for 20 hours with or without BM-MSC either in direct contact or in Transwell, at ratios ranging from 1000:1 to 10:1, in the absence or presence of 100 μg/ml poly(I:C) or 100 ng/ml LPS. **(A):** Bars indicate the survival levels of PMN after coculture. PMN that were double negative by the Annexin-V/PI method (see Materials and Methods section) were considered as viable: their percentage is reported as percentage of the total. **(B):** PMN were analyzed for CD16 expression by FACS analysis (see Materials and Methods section). Bars refer to the percentage of PMN maintaining a high level of CD16 expression (CD16^{high} PMN, see Materials and Methods section). **(C):** PMN were analyzed for CD11b expression by FACS analysis (see Materials and Methods section). Bars indicate the geometric mean of fluorescence intensity ± SD of CD11b in PMN. Results are expressed as mean ± SD ($n \geq 12$ in all cases). p values < .05 were considered as statistically significant (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cell; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; PMN, neutrophils; poly(I:C), polyinosinic:polycytidylic acid.

BM-MSC-Conditioned Supernatants Virtually Reproduce All the Effects Observed in PMN:BM-MSC Cocultures

By culturing PMN in supernatants from either resting, poly(I:C)-, or LPS-stimulated BM-MSC, the same effects detected under Transwell cocultures were substantially observed in terms of both PMN survival and phenotypic changes (Fig. 4). However, conditioned media from poly(I:C)-treated BM-MSC were slightly more efficient than supernatants from LPS-stimulated BM-MSC in enhancing the percentage of viable, CD16^{high} and CD11b+ PMN (Fig. 4). Note that PMN were pretreated with a specific TLR4 blocking antibody [38] before their incubation with the supernatant from LPS-stimulated BM-MSC. The efficacy of TLR4 blocking in fully neutralizing the effects mediated by LPS is shown in Supporting Information Figure 8. Overall, these data confirm that soluble factors contribute to mediate the modulatory effects exerted on PMN by resting or TLR-activated BM-MSC.

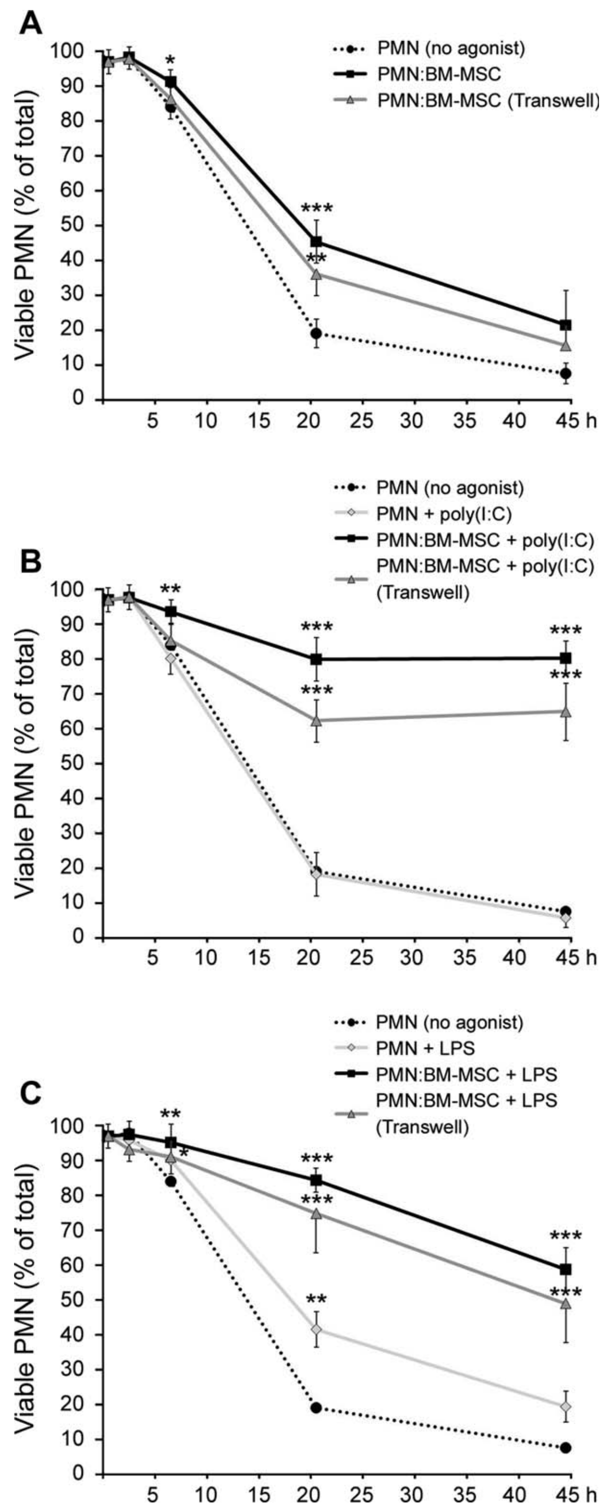


Figure 2. Time course analysis of the viability of neutrophils (PMN) during their coculture with resting and activated bone marrow-derived mesenchymal stromal cells (BM-MSCs). PMN were cocultured with or without BM-MSC as detailed in the legend of Figure 1 (PMN:BM-MSC coculture ratio being always 10:1) and harvested at different time points (0, +2, +6, +20, and +44 hours) to measure their viability. Double-negative PMN according to the Annexin-V/PI method were considered as viable, and their percentage is reported as percentage of the total. Results are expressed as mean ± SD ($n = 3$). Panel **(A)** displays the survival in cultures without Toll-like receptor (TLR) agonists, whereas Panels (B) and **(C)** display cultures with the addition of poly(I:C) and LPS, respectively. Results was statistically compared with PMN cultures when carried out without TLR agonists, and p values < .05 were considered as statistically significant (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cell; LPS, lipopolysaccharide; PMN, neutrophils; poly(I:C), polyinosinic:polycytidylic acid.

IL-6, IFN-β, and GM-CSF are Primarily Responsible for the Effects on PMN Survival and CD11b Expression Mediated by BM-MSC

We then measured a number of cytokines known to be involved in PMN survival and activation, including IL-6, IL-8/CXCL8, IFN-α, IFN-β, IFN-γ, G-CSF, GM-CSF, and TNF-α; among them, only IL-6 and IL-8/CXCL8 were detectable in supernatants harvested from resting BM-MSC cultured for 24 hours (Table 1). The latter molecules were present at much higher levels in the supernatants from both poly(I:C)- or LPS-stimulated BM-MSC, both of which also contained GM-CSF (Table 1). Furthermore, while G-CSF, IFN-α, IFN-γ, or TNF-α were never detectable, IFN-β was specifically measurable only in poly(I:C)-conditioned medium (Table 1). The presence of IFN-β in poly(I:C)-conditioned supernatants was consistent with the evidence that TLR3-activated BM-MSC displayed IRF3 dimers and delayed STAT1 tyrosine phosphorylation [39] (Supporting Information Fig. 9). On the other hand, no IRF3 or STAT1 activation occurred in LPS-stimulated BM-MSC (data not shown), which displayed a marked activation of both nuclear factor-κB and mitogen activated protein kinase signaling pathways, as expected (Supporting Information Fig. 9).

To identify which of the cytokines detected were responsible for PMN survival and activation under coculture conditions, we subsequently cultured PMN in the presence of specific neutralizing antibodies against IL-6R, type I IFN-R, GM-CSF, IL-8/CXCL8 and, as negative controls, G-CSF, IFN, and the related isotype matched controls. Both the protective effect on PMN survival and the induction of higher CD11b expression by untreated MSC were neutralized by the anti-IL-6R mAbs (data not shown), thus confirming previous findings [25]. By contrast, the effects

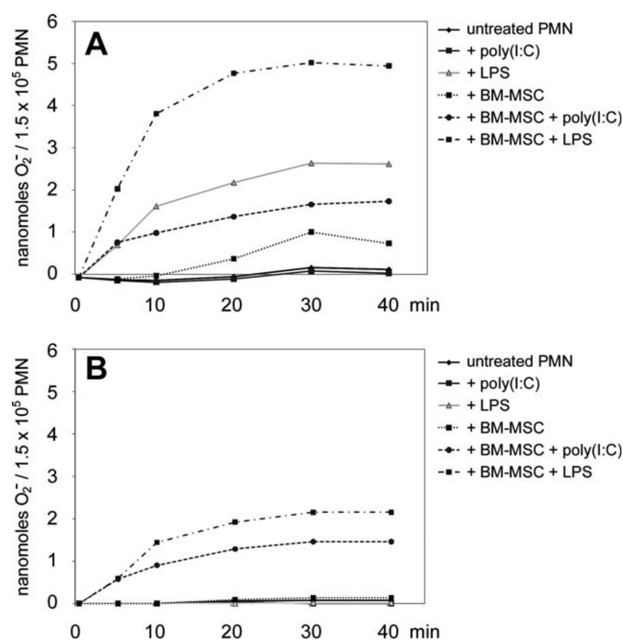


Figure 3. Coculture with bone marrow-derived mesenchymal stromal cell (BM-MSC) primes neutrophils (PMN) for increased respiratory burst. PMN were cocultured for either 20 hours **(A)** or 72 hours **(B)** with or without BM-MSC at 10:1 ratio, in the absence or presence of 100 μg/ml poly(I:C) or 100 ng/ml LPS. Superoxide anion (O_2^-) production in response to 100 nM fMLF was then estimated by the cytochrome C reduction assay of triplicate samples. Absorbance at 550/468 nm was recorded every 5 minutes for the times shown. O_2^- production was calculated in nanomoles per 1.5 $\times$ 10^5 PMN per minute using 24.5 mM as extinction coefficient. Each panel of the figure shows a representative experiment out of three performed with similar results. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cell; LPS, lipopolysaccharide; PMN, neutrophils; poly(I:C), polyinosinic:polycytidylic acid.

obtained with supernatants from poly(I:C)-treated BM-MSCs were almost completely neutralized by the simultaneous use of anti-IL-6R, anti-GM-CSF, and anti-type-I IFN-R mAbs, which, if used individually, produced only a limited, partial block (Fig. 5). Interestingly, the effects of supernatants from LPS-treated BM-MSC were significantly reverted, although not completely, by anti-GM-CSF mAbs only, all the other antibodies being totally ineffective (Fig. 5). On the other hand, the addition of IL-6 and IFN-β to supernatants harvested from LPS-stimulated BM-MSC at approximately the same concentrations measured in supernatants from poly(I:C)-activated BM-MSC improved their effects in terms of PMN survival and CD16/CD11b expression, even to matching the levels observed by using poly(I:C)-conditioned media (data not shown). Isotype controls did not exert any effect under all stimulatory conditions (data not shown). These latter experiments confirm the key roles of BM-MSC-derived IL-6 and IFN-β, in combination with BM-MSC-derived GM-CSF, in determining the effects exerted after TLR3 stimulation in PMN:BM-MSC cocultures.

MSC from Thymus, Spleen, and Adipose Tissue Mimic the Effects Exerted by BM-MSC on PMN

MSCs expanded from tissues other than BM (i.e., thymus, spleen, and adipose tissue) were used to assess whether the TLR3- and TLR4-dependent effects observed with BM-MSC were general mechanisms of MSC populations. We found that all types of MSCs behaved like BM-MSC in terms of either their protective effect on PMN survival (Supporting Information Fig. 10A) or immunophenotypic changes (Supporting Information Fig. 10B, 10C), both at resting conditions and after TLR3 or TLR4 engagement, at least at the 10:1 PMN:MSC coculture ratio.

TLR3 or TLR4 Activation of BM-MSC Previously Primed by IFN-γ and TNF-α Induces Potent Prosurvival Effects and Immunophenotypic Changes in PMN

BM-MSCs are known to change their biological behavior and pattern of cytokine production when exposed to cytokines mimicking an inflammatory microenvironment [40]. To evaluate the role of BM-MSC “inflammatory priming” in our model, we cocultured PMN and BM-MSC in the presence of poly(I:C) or LPS after previously exposing BM-MSC to IFN-γ and TNF-α for 48 h. Under these new conditions, we observed that “primed” BM-MSC was more potent than untreated BM-MSC in prolonging PMN survival and in upregulating PMN CD16/CD11b expression (Fig. 6). Remarkably, we also observed that PMN survival and CD16/CD11b expression were further increased by the addition of poly(I:C) and LPS to the cocultures (Fig. 6).

Discussion

In this study, we aimed at exploring under coculture conditions whether TLR-activated MSC could exert different modulatory effects on PMN as compared to untreated MSC under coculture conditions. We specifically focused our attention on BM-MSC stimulated with poly(I:C) to detect direct BM-MSC-mediated effects toward human PMN, which do not express TLR3 and do not respond to its ligands [34, 35]. As control, we analyzed LPS-stimulated PMN in coculture with BM-MSC, always carefully considering that both BM-MSC and PMN express the functional TLR4 [16, 27, 32, 33]. TLRs belong to the pattern recognition receptor system that has multiple and pleiotropic functions, including the triggering of PMN during inflammation [34]. Also BM-MSC express several TLRs, capable of activating specific responses [16, 17, 27, 32, 33]; for instance, while TLR2 maintains BM-MSC in their undifferentiated state, without affecting their immunomodulatory properties [41], both TLR3 and TLR4 influence their response to stress and migration [27, 32], as well as regulate their immunomodulatory effects toward activated T lymphocytes [16, 17, 42]. In addition, TLR3 and/or TLR4 engagement enhances BM-MSC production of IL-1β, IL-6, and chemokines, such as IL-8/CXCL8, IP10/CXCL10, monocyte chemotactic protein-1, and CCL516, 27, 32, 33, 41. Finally, TLR3 triggering seems to mediate, under specific conditions, the MSC polarization toward the inhibitory phenotype, while TLR4 activation would drive MSC toward the opposite proinflammatory status [17].

Herein, using highly purified preparations of human PMN, we confirmed that in the absence of stimuli BM-MSC significantly prolong PMN survival in an IL-6-dependent manner, as previously described [25]. However, in our experiments such antiapoptotic effect was statistically significant only at a 10 times (one log) higher coculture ratio and lost statistical significance after 44 hours of coculture. On the other hand, there were no signs of PMN activation following coculture with resting BM-MSC, as previously described [25]. In particular, we did not observe any change either in the levels of PMN CD11b expression or in their respiratory burst capacity.

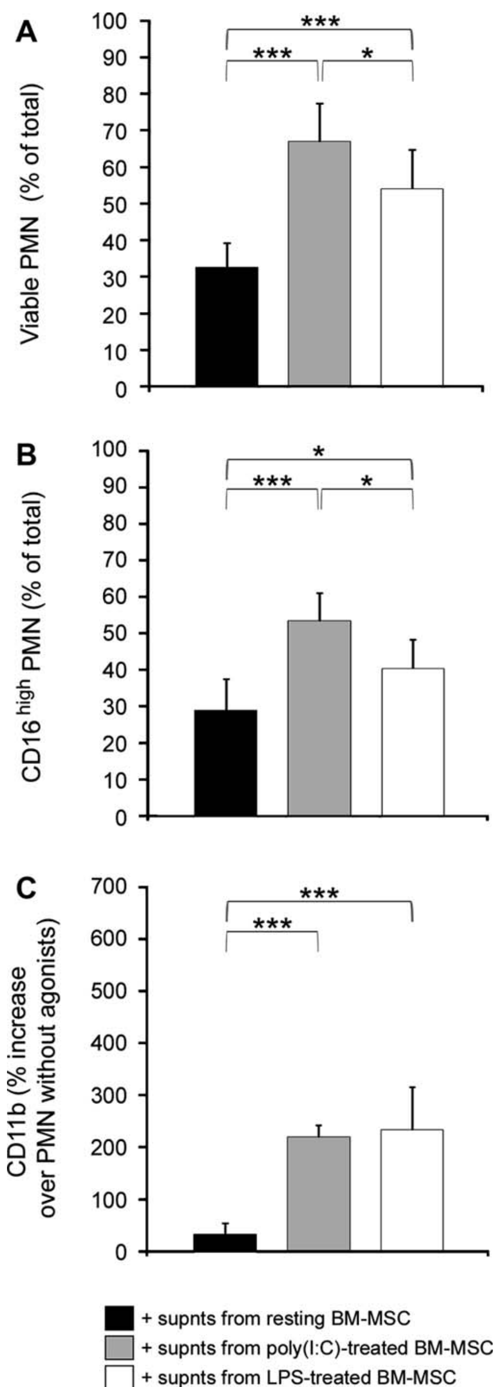


Figure 4. Effects of bone marrow-derived mesenchymal stromal cell (BM-MSC)-conditioned supernatants on neutrophil viability and expression of CD16 or CD11b. BM-MSC were cultured for 24 hours with or without 100 μg/ml poly(I:C) or 100 ng/ml LPS, before collecting their supernatants. Freshly isolated neutrophils (PMN) were then incubated with the various BM-MSC-conditioned supernatants and analyzed after 20 hours for viability **(A)**, CD16 **(B)**, and CD11b **(C)** expression. Bars in panel **(B)** represent the percentage of $CD16^{high}$ PMN in the various conditions; bars in panel **(C)** express CD11b expression as percentage of increase over the level observed in the case of PMN cultured alone without Toll-like receptor agonists. All results are expressed as mean ± SD ($n = 6$). *, $p < .05$; ***, $p < .001$. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cell; LPS, lipopolysaccharide; PMN, neutrophils; poly(I:C), polyinosinic:polycytidylic acid.

Furthermore, no cytotoxic effect mediated by PMN toward the MSC monolayer during coculture was observed (F.M. and M.P., unpublished observations).

Strikingly, poly(I:C)-activated BM-MSC exerted a significantly greater protection of PMN from apoptosis than resting BM-MSC; in fact, this effect was evident at lower (i.e., 100:1) PMN:BM-MSC ratio and lasted up to 44 hours of coculture. In addition, poly(I:C)-activated BM-MSC strongly enhanced PMN respiratory burst ability and CD11b expression. Similar effects were detected in PMN cocultured with BM-MSC in the presence of LPS, which was apparently even more powerful than poly(I:C) in activating BM-MSC prosurvival effects. However, when comparing coculture data with those from PMN-only cultures, this advantage was partially related to the direct effect of LPS on PMN survival and immunophenotype. Thus, TLR3-stimulated BM-MSC appeared more efficient than TLR4-stimulated BM-MSC, as confirmed also by the comparative experiments of PMN culture in supernatant from either LPS- or poly(I:C)-triggered BM-MSC. MSC obtained from different tissues, such as thymus, spleen, and subcutaneous adipose tissue, displayed the same effects of BM-MSC in terms of PMN survival and phenotypic pattern, thus strengthening the concept that TLR3 or TLR4 might regulate the interactions in different tissues between stromal cells and recruited PMN during inflammatory reactions.

Similar effects were obtained either under Transwell conditions or by culturing PMN in supernatants from BM-MSC previously exposed to poly(I:C) or LPS for 24 hours, thus suggesting that soluble factors were involved. Although IFN-α, TNF-α, G-CSF, IFN-γ, and IFN-α could have been important candidates to mediate the observed effects [19], none of them were detected in any of the BM-MSC-derived supernatants; by contrast, high levels of both IL-6 and, to minor extent, IL-8/CXCL8 were found in supernatants from resting BM-MSC. These cytokines were even more concentrated in poly(I:C)- or LPS-derived supernatants, as previously reported [16, 32, 33]. Furthermore, we detected significant amounts of GM-CSF in supernatants from both poly(I:C)- and LPS-stimulated BM-MSC, whereas IFN-β was found only in samples harvested from TLR3-treated BM-MSC. In the latter regard, the activation of IRF3 as well as the tyrosine phosphorylation of STAT1 detected in BM-MSC exposed to poly(I:C), but not to LPS, is consistent with a specific production of IFN-β only after TLR3 engagement. Subsequent experiments with specific neutralizing antibodies showed that poly(I:C)-stimulated BM-MSC promote PMN survival and CD11b upregulation almost completely through the combined action of IL-6, IFN-β, and GM-CSF, while each single cytokine exerts only a partial effect. By contrast, the effects exerted by LPS-stimulated BM-MSC supernatants could be only partially blocked by anti-GM-CSF mAbs; in addition, anti-IL-6 mAbs alone were very poorly effective and anti-type-I IFN-R, anti-G-CSF, anti-IFN-γ, and anti-IL-8/CXCL8 mAbs did not exert any change. The latter data complement and extend the findings recently described by Brandau et al. [27], showing that supernatants harvested from parotid-derived MSC exposed to LPS for 4 hours contained many different inflammatory cytokines and were capable of delaying PMN apoptosis. These authors also quantified large amounts of G-CSF, TNF-α, and IFN-γ and they consequently assumed these cytokines as responsible for the observed effects on PMN, without however formally proving their hypothesis. As mentioned, we were unable to detect G-CSF, TNF-α, or IFN-γ in supernatants of LPS-stimulated preparations of BM-MSC, even by means of high-sensitivity ELISA (see Materials and Methods section). Accordingly, the expression by PMN of CD64, a marker well-known to be

Table 1. Cytokine released by bone marrow-derived mesenchymal stromal cells activated by poly(I:C) or LPS

Cytokine	No agonist	+Poly(I:C)	+LPS
IL-6 (ng/ml)	1.3 ± 0.19	22.1 ± 0.62	14.2 ± 0.91
IL-8/CXCL-8 (pg/ml)	48.5 ± 21.2	2,873.3 ± 6.3	2,846.1 ± 4.4
IFN-α (U/ml)	n.d.	n.d.	n.d.
IFN-β(U/ml)	n.d.	5.964 ± 0.2	n.d.
IFN-γ (U/ml)	n.d.	n.d.	n.d.
G-CSF (pg/ml)	n.d.	n.d.	n.d.
GM-CSF (pg/ml)	n.d.	22.27 ± 19.5	21.35 ± 7.3
TNF-α (pg/ml)	n.d.	n.d.	n.d.

IL-6, IL-8/CXCL-8, interferon-α (IFN-α), IFN-β, IFN-γ, G-CSF, GM-CSF, and tumor necrosis factor-α were measured by specific enzyme-linked immunosorbent assay in supernatants harvested from bone marrow-derived mesenchymal stromal cells (BM-MSC) cultured for 24 hours in the absence or presence of either 100 μg/ml poly(I:C) or 100 ng/ml LPS. Concentration levels are expressed as mean ± SD calculated from three sets of experiments performed with BM-MSC expanded from two independent healthy donors. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; n.d., not detectable; TNF-α, tumor necrosis factor-α.

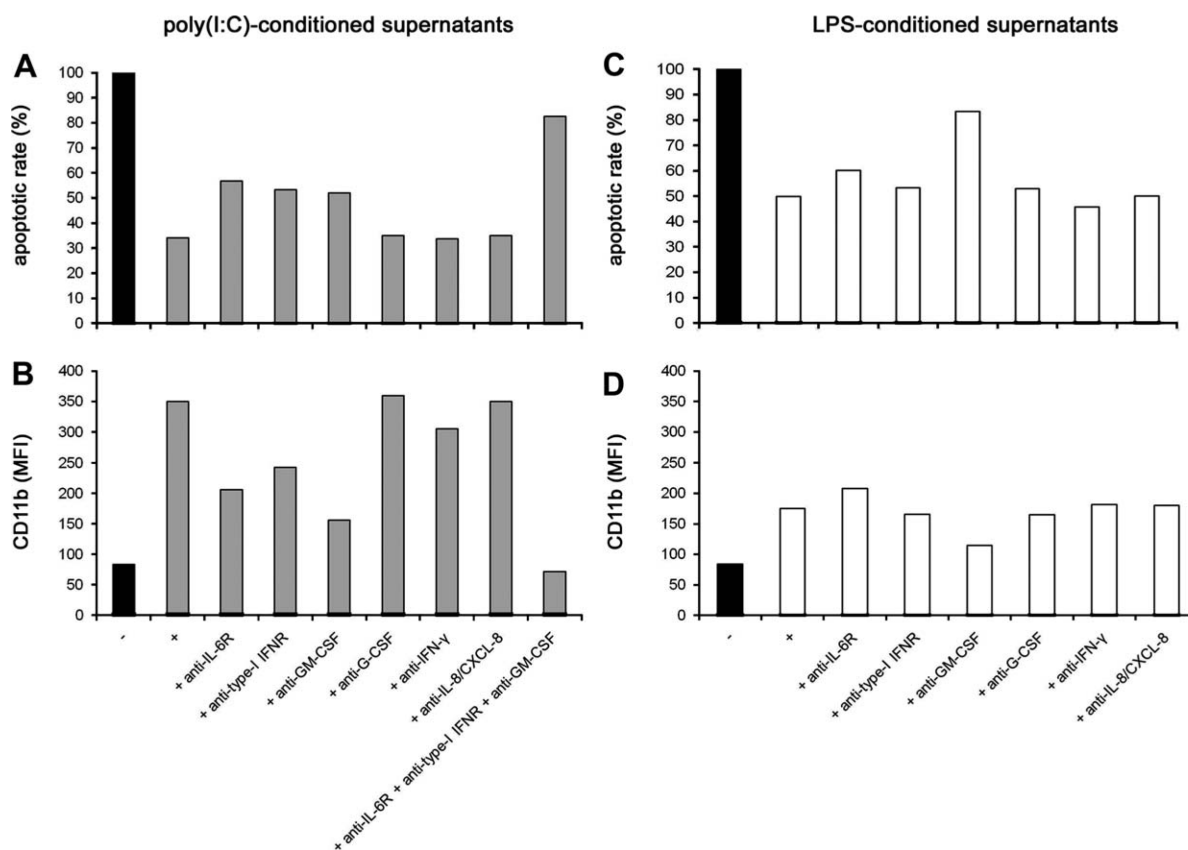


Figure 5. Poly(I:C)-activated bone marrow-derived mesenchymal stromal cells (BM-MSCs) modulate neutrophil apoptosis and CD11b expression through the release and combined action of IL-6, GM-CSF, and interferon-β (IFN-β). **(A–D):** Human neutrophils (PMN) were cultured for 20 hours in supernatants collected from poly(I:C)- **(A, B)** or LPS-stimulated BM-MSC **(C, D)**, in the presence or absence of 10 μg/ml neutralizing monoclonal antibodies (mAbs) (or appropriate isotype controls, not shown) directed toward IL-6R, type-I IFN-R, GM-CSF, G-CSF, IFN-γ, and IL-8/CXCL8, either alone or in combination. In all conditions involving the use of supernatants from LPS-activated BM-MSC **(C, D)**, PMN were preincubated for 30 minutes with anti-Toll-like receptor-4 (TLR4) mAbs (see Materials and Methods section), before further culture. The degree of apoptosis **(A, C)** was compared with what was observed in PMN culture performed in regular medium (see Materials and Methods section) without TLR agonists (apoptotic rate); the expression of CD11b **(B, D)** was measured as geometric mean fluorescence intensity. One representative experiment out of two performed with BM-MSC expanded from two independent healthy donors, with similar results, is depicted. Black column (–): untreated cells; first gray column (+): cells cultured in conditioned supernatants with no neutralizing antibodies. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; poly(I:C), polyinosinic:polycytidylic acid.

upregulated after exposure to IFN-γ [43], was never increased in PMN under any of the coculture conditions (data not shown). In addition, data supporting the production of either IFN-γ or TNF-α by BM-MSC expanded from healthy donors are still controversial, at best [32]. Nevertheless, in our opinion, these data discrepancies could likely reflect a different

status of MSC activation due to their isolation from healthy rather than pathological microenvironments.

To our knowledge, this is the first study reporting modulatory effects on PMN survival and activation exerted by MSC via TLR3 activation. As poly(I:C) mimicks in vitro double-stranded viral RNA (the natural ligand of TLR3) [39], a functional cross-talk between MSC and PMN might occur in vivo during the occurrence of defense mechanisms against viral infections. In fact, recent publications have pointed out a potential role played by PMN during antiviral responses [35, 44]. This role, however, still remains underestimated, particularly when considering the strict cooperation existing between PMN and NK cells [45]. By indicating, for the first time, the occurrence of functional changes in PMN after the triggering of TLR3 on MSC, our study suggests a novel and previously unknown stroma-dependent activation mechanism. By the modulation of their cytokine production, TLR3 might activate tissue-resident MSC to recruit (by chemokines such as IL-8/CXCL8) and activate PMN to face viral infections. In fact, according to the observations made in this study, as well as to the results by Waterman et al. [17] suggesting that human MSC polarize toward an inhibitory phenotype (MSC2) following TLR3 activation [17], one could speculate that TLR3-activated MSC maintain an efficient PMN-dependent innate response, while persistently suppressing T-cell activation. Future in vivo studies are necessary to better characterize this new biological mechanism.

Moreover, TLR3-activated MSC might influence PMN behavior also in other pathological conditions, such as tissue necrosis. In fact, endogenous double-stranded RNA may form during tissue necrosis as a result of the spontaneous involution of highly repetitive nucleotidic sequences of RNA strands [46, 47] that, in turn, may activate TLR3 in human dendritic cells [47], as well as in murine PMN and macrophages [46]. Thus, tumor-associated MSC and stromal cells could be similarly activated through TLR3 in those malignancies characterized by foci of internal necrosis, thereby sustaining the recruitment and the activation of tumor-infiltrating PMN via the production of TLR3-induced IL-8/CXCL8 and CCL5 [16, 27, 32, 33]. Additionally, the demonstration that TLR3 ligation on MSC triggers a cascade of events that ultimately favor a prolonged PMN survival and enhanced respiratory burst ability might also have negative implications for the therapeutic use of MSC. For instance, the controversial results obtained by injecting MSC into the inflamed joints of patients affected by rheumatoid arthritis [48] might be explained by the shift of MSC toward an unexpected proinflammatory, PMN-supporting phenotype upon in vivo stimulation of their TLR3. In fact, RNA released from necrotic synovial fluid cells has already proved capable of activating fibroblasts from rheumatoid arthritis synovial membrane via TLR3 [49]; a

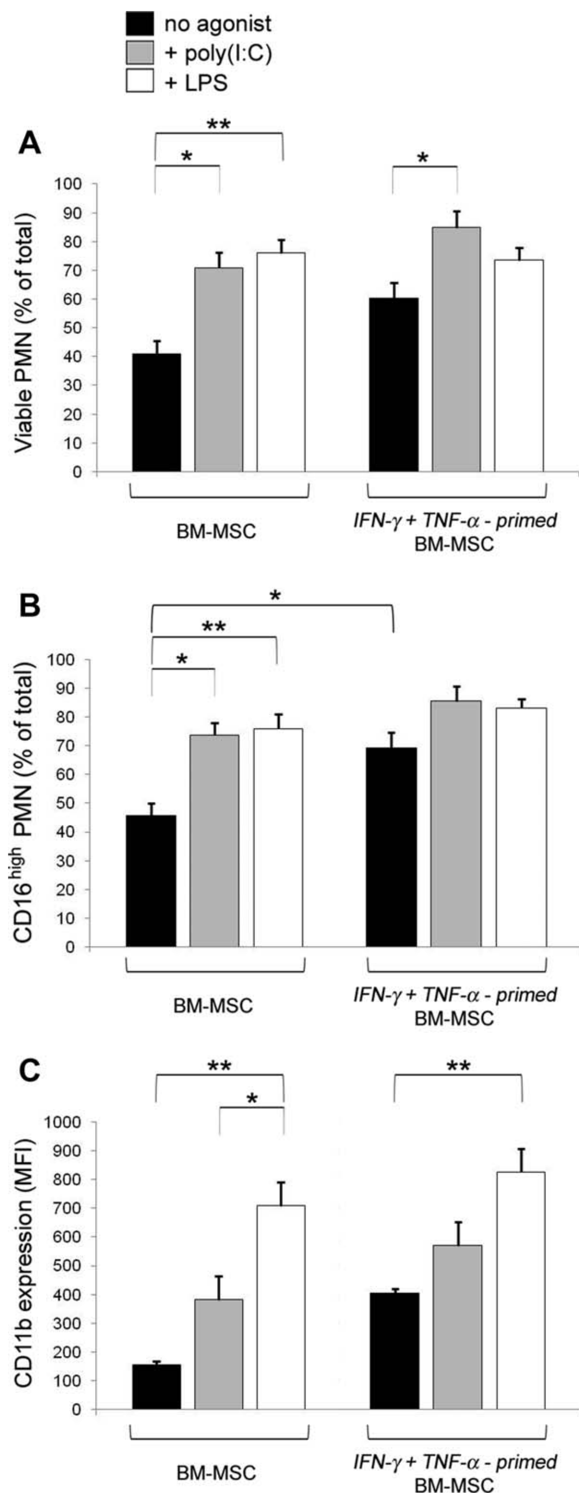


Figure 6. Toll-like receptor-3 (TLR3) or TLR4 activation of bone marrow-derived mesenchymal stromal cells (BM-MSCs) previously primed by interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) exert a protective neutrophil prosurvival effect and induce neutrophil immunophenotypic changes. BM-MSCs were exposed to 200 IU/ml of IFN-γ and 1,650 IU/ml of TNF-α for 48 hours before their coculture with neutrophils (PMN) in the presence of 100 μg/ml poly(I:C) or 100 ng/ml LPS for further 20 hours. **(A):** Bars indicate the survival levels of PMN after coculture. PMN that were double negative by the Annexin-V/PI method (see Materials and Methods section) were considered as viable: their percentage is reported as percentage of the total. **(B):** PMN were analyzed for CD16 expression by FACS analysis (see Materials and Methods section). Bars refer to the percentage of PMN maintaining a high level of CD16 expression (CD16^{high} PMN, see Materials and Methods section). **(C):** PMN were analyzed for CD11b expression by FACS analysis (see Materials and Methods section). Bars indicate the geometric mean of fluorescence intensity $\pm$ SD of CD11b in PMN. Results are expressed as mean $\pm$ SD (n = 3). p values < .05 were considered as statistically significant. *, p < .05; **, p < .01; ***, p < .001. Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; INF, interferon; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; PMN, neutrophil; poly(I:C), polyinosinic:polycytidylic acid; TNF-α, tumor necrosis factor-α

similar phenomenon could occur following stimulation by autoantigens and/or endogenous ligands.

Conclusion

Our data add new information on the notion of a presumed functional MSC polarization induced by TLR3 and TLR4 triggering. In fact, a new paradigm for MSC has been recently proposed on the basis of the analogy with the functional status of monocytes/macrophages [17]; in particular, that TLR4-primed MSC would exhibit a mostly proinflammatory profile with increased levels of molecules like IL-6, IL-8, or TGF-β (and thus named as MSC1), while TLR3-primed MSC would develop the characteristics of immunosuppressive cells producing IL-10, IDO, and PGE2 (and thus named as MSC2) [17].Our data are partially in contrast with this paradigm, as they show that MSC of different tissue origin, in response to TLR3 triggering, may normally become proinflammatory by supporting the survival and function of PMN through the release of IL-6, IFN-β, and GM-CSF. Such effects should physiologically evolve into a correct immune response aimed to eliminate the danger signals that engage TLR3. However, if dysregulated, the process could lead to the development of chronic inflammation and autoimmune disorders. Thus, the role of the persistent stimulation of tissue-resident MSC via TLR3 and TLR4 under these conditions will have to be clarified by future studies.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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