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ROLE OF TOLL-LIKE RECEPTORS-3 AND -4
IN THE INTERACTIONS BETWEEN NEUTROPHILS AND
MESENCHYMAL STROMAL CELLS

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SUMMARY

Bone marrow-derived mesenchymal stromal cells (BM-MSC) are stromal precursors endowed with extensive immunomodulative properties. In this study, we aimed to assess whether Toll-like receptor(TLR)3- and TLR4-activated BM-MSC influence human neutrophil responses under coculture conditions. We show that TLR3 triggering by poly(I:C) dramatically amplifies, in a more significant manner than TLR4 triggering by LPS, the antiapoptotic effects that resting BM-MSC constitutively exert on neutrophils under coculture conditions. In addition, TLR3- and TLR4-activated BM-MSC enhance respiratory burst ability and CD11b expression by neutrophils. The coculture in the absence of cell contact and the incubation of neutrophils 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 neutrophils by TLR3-activated BM-MSC are mediated by the combined action of IL-6, IFN- β and GM-CSF, while those exerted by TLR4-activated BM-MSC mostly depend on GM-CSF. MSC isolated from thymus, spleen and subcutaneous adipose tissue behave similarly.

Therefore, our data highlight a novel mechanism by which MSC sustain and amplify the functions of neutrophils in response to TLR3- and TLR4-activation and may consequently contribute to inflammatory disorders.

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INTRODUCTION

TOLL-LIKE RECEPTORS:

Definition and characterization

Toll-like receptors (TLRs) are transmembrane receptors mediating the activation and functions of several cells of the innate and acquired immune system, including polymorphonuclear (PMN) neutrophils¹⁻³. By recognizing highly-diffused pathogen-associated molecular patterns (PAMPs) present in viruses, bacteria and other pathogens, they provide a fundamental trigger for early inflammation and the subsequent immune response.

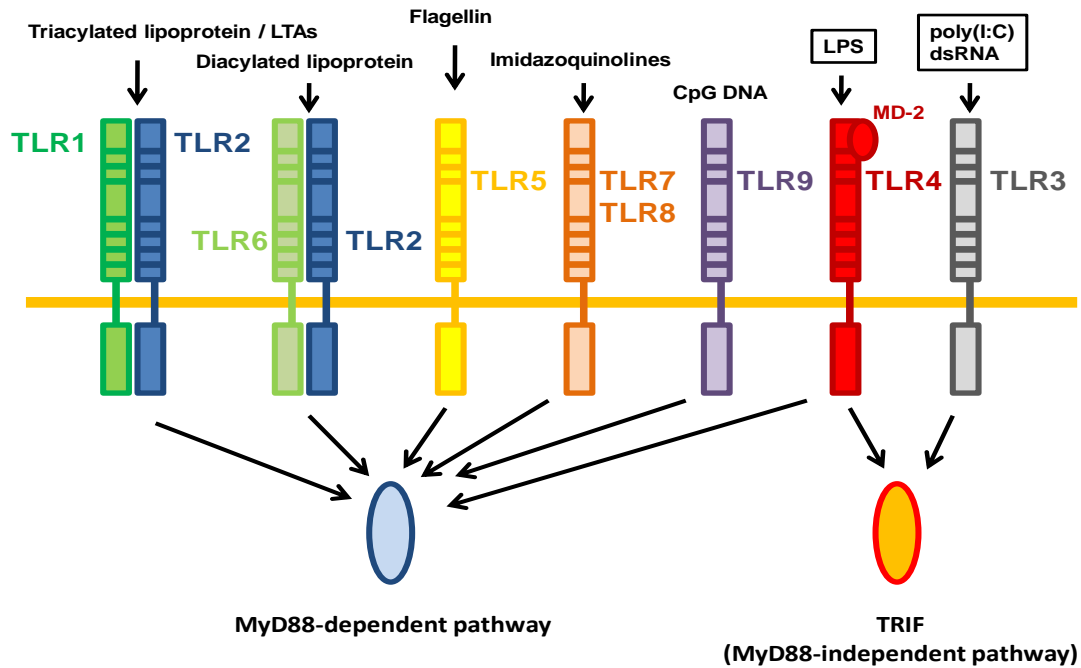
Originally, TLRs were discovered by means of mouse mutants (and a classic genetic approach) as the homologues in mammals of *Drosophila* Toll molecule⁴⁻⁵. In fruitflies, Toll was initially identified as one of the genes responsible for the differentiation of ventral and dorsal structures during embryo development⁵; as later studies have demonstrated, it is actually a very ancient, phylogenetically conserved molecule modulating also the interactions of cells with their microenvironment and, especially, the initial response to pathogens (e.g. in *Drosophila*, fungal infections)⁶. Their homologues can be found in plants, as well, where several disease resistant genes encode proteins characterized by the presence of the *Toll/IL-1 receptor* intracellular domain (TIR), typical of both TLRs and members of the IL-1R family. Their precise mechanisms of function in plants, however, are very different from animals, and largely still unknown⁷. In mammals, Toll-like receptors are not involved in embryo development, as mice lacking TLR4 develop normally^{2,7}. Instead, they belong to the

family of pathogen-associated molecular patterns (PAMP)-recognizing receptors, capable of activating several cells of the innate immune system in recognition of molecular patterns featured by non-self, potentially dangerous organisms^{4,8-10}.

Structurally, TLRs shows high similarity with that of the IL-1 receptor family: their cytoplasmic portion, i.e. the TIR domain, originates from a common genetic precursor. Despite this shared evolution, though, the extracellular part of the two is unrelated, as TLRs bear leucine-rich repeats (LRRs), while IL-1 receptors possess an Ig-like domain^{4,8}. TLRs are functional multimers: most of them are homomeric, but some are instead heteromeric, e.g. TLR2, which strictly associates with TLR1 or TLR6. TLR4, finally, forms even more complex structures together with coreceptors, such as CD14 and the LPS-binding subunit MD2^{2,4,8}.

Ten different TLRs have been discovered, so far, in humans (**Figure 1**)¹¹; each of them is characterized by sensitivity to specific molecular patterns easily found in pathogens¹⁰. Overall, TLRs that are usually exposed on the surface of the cells, i.e. TLR1, -2, -4, -5, -6 are sensitive to bacterial-derived molecules, such as either bacterial lipoproteins and lipoteichoic acids (LTA) (TLR1/TLR2 and TLR6/TLR2), or LPS (TLR4), or flagellin (TLR5)^{7,10}. TLRs more commonly present in the cell cytoplasm, i.e. TLR3, -7, -8, -9 are usually responsible for reactions to molecules derived from intracellular pathogens and viruses, such as double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and -8), or unmethylated CpG DNA fragments (TLR9)⁷⁻¹⁰. No specific ligand for TLR10 has been identified, as yet.

FIGURE 1. Toll-like receptors in humans.



(modified from Takeda et al., 2004)

All Toll-like receptors (TLR) recognize molecular pattern present in microbial components. TLR2 works in heterodimers together with either TLR1 or TLR6 for the recognition of microbial lipopeptides and lipotheicoic acids (LTAs). TLR4 recognizes lipopolysaccharides (LPS), together with mediators such as MD-2 or co-receptors such as CD14 (*not shown*). TLR3 and TLR9 recognize viral-related nucleic acids sequences, i.e. double-stranded RNA (dsRNA) and CpG DNA, respectively. TLR5 is the receptor for flagellin. All TLRs except TLR3 activate the MyD88-dependent pathway through the coupling protein MyD88. TLR3 and, partially, TLR4 act downstream by a MyD88-independent pathway which uses TRIF (*TIR-domain containing adaptor inducing IFN- β*) as primary coupling protein.

TLRs are widely expressed by the main cells of the innate and adaptive immune system, including monocyte/macrophages⁹⁻¹⁰, polymorphonuclear leukocytes^{1,3}, dendritic cells¹²⁻¹⁴, T and B lymphocytes^{13,15-17}, NK cells¹⁶. In all vertebrates, and particularly in mammals, TLRs are deeply involved in the far more complex mechanisms that enable the discrimination of the *self* from *non-self* by the immune system². In fact, their activation during organ transplantation can prevent the development of immune tolerance towards the transplant¹⁸. In monocytes, and in most leukocytes, as well, their challenge by adequate stimuli results in the activation of the defensive mechanisms provided by the cells and in the release of cytokines, chemokines and other powerful proinflammatory mediators. Although TNF- α , IL-1 β and IL-6 are usually cited as example, chemokine genes actually show the strongest gene induction upon TLR triggering⁷.

Besides their PAMP-recognizing abilities, TLRs also serve other very different functions depending on the cell types which express them⁹⁻¹⁰. For instance, TLR3, besides being a typical “intracellular” TLR, is expressed both intracellularly and on the cell surface by human fibroblasts, epithelial cells¹⁹⁻²⁰ and human mesenchymal stromal cells²¹⁻²³, where it promotes various functions, mostly related to cell sensitivity to microenvironmental changes and the triggering of defensive responses, but still largely unknown. Epithelial cells at potential sites of entry, including the skin and the respiratory, intestinal and genitourinary tracts, all express TLRs, again with very different cell-specific functions⁷. Endothelial cells and vascular smooth muscle cells, for instance, are sensitive to potential infective agents by their own TLRs, and, in the presence of signs of an infection, mediate the very early defense by switching capillary networks to a proinflammatory phenotype²⁴.

Further increasing the overall complexity of TLR networks, it is worth to note that the expression pattern of TLRs can in some cases vary even when considering the same cell type: for instance, monocytes and tissue macrophages can significantly up- or downregulate their expression of TLRs and, thus, their sensitivity to stimuli depending on the conditions of the microenvironment and the presence of cytokines, chemokines and other pro-inflammatory molecules⁷.

TLRs also serve as an important link between innate and adaptive immunity through actions on T cells^{15,25-26} and particularly on dendritic cells¹⁴⁻¹⁵. DC maturation into active antigen-presenting cells (APCs), capable of exerting a proper T cell response, is highly dependent on TLR stimulation by pathogen-derived molecules acquired by phagocytosis¹⁴. Moreover, TLR3 activation on several cell types results in highly efficient type-I interferon (IFN- α/β) production. Besides their antiviral properties, both interferons play an important role in many phases of a typical immune response, such as the proliferation of memory T cells, the inhibition of T cell apoptosis, the secretion of IFN- γ and the differentiation of B cells into plasma cells¹⁵. Finally, IFN- α and IFN- β also modulate the activation of NK cells¹⁵.

Even though TLRs are primarily thought to have evolved as sensors of exogenous stimuli, several endogenous ligands have been characterized, especially in paraphysiological and pathological conditions. They may represent products from damaged host cells, but are still unknown in many cases. A potential example are small pieces of repetitive mRNA released from necrotic tissue and folded in short double-stranded sequences, which have been demonstrated to be able to activate TLR3 in several mouse models of enterogenic sepsis²⁷⁻²⁸. In these animals, TLR3 activation occurs in the absence of exogenous viral stimulus, and the block of its signaling,

obtained either by using TLR3-deficient mice or by neutralizing anti-TLR3 mAbs, was associated with significantly lower levels of tissue injury and lower sepsis-induced mortality²⁸.

Besides necrotic mRNA, other potential endogenous ligands for TLRs (reviewed in Parker *et al.*⁷) include: antimicrobial molecules such as defensins (recognized by TLR2); the high mobility group box 1 (HMGB1) protein (a potential ligand for both TLR2 and TLR4); proteins from damaged tissue, such as heat-shock protein B8, fibrinogen, surfactant protein A (all sensed by TLR4); products of tissue matrix digestion, such as fibronectin extra domain A and hyaluronic acid oligosaccharides (*via* TLR4). TLR2 has shown sensitivity also to reactive oxygen species, and oxydative stress was demonstrated to influence cardiomyocyte behaviour *via* TLR2²⁹.

Despite this, in many cases, regardless of consistent evidence of TLR activation in the course of several diseases, the actual endogenous trigger responsible for it *in vivo* is still unidentified. One such example is asthma: TLRs play a crucial role in the development and worsening of the disease, as contaminating endotoxin is routinely inhaled with air, and TLRs are expressed either in tissue-resident epithelial cells and alveolar macrophages, as well as in airways-infiltrating monocytes and bronchial smooth muscle cells³⁰. By acting on TLRs sensitivity, viruses like respiratory syncytial virus (RSV) and influenza A can significantly worsen asthma, along with other respiratory diseases. For instance, sensitization to viral and bacterial products, a common consequence of viral infections in asthmatic patients, occurs *via* up-regulation of TLR3 and -4 in several types of bronchial cells³¹⁻³².

Another example of diseases involving TLR activation in their pathogenesis, even without clearly identified endogeneous ligands, is cancer. TLR signaling has been investigated as pivotal in the development of a chronic inflammatory process leading up

to gastrointestinal cancer in its early stages³³, as well as in the development of chemoresistance by different types of malignancies³⁴⁻³⁵. At the same time, the cytokines produced, as a consequence of TLR stimulation, by either tumor-associated stromal cells and tumor-infiltrating leukocytes have been addressed as powerful inducers favouring tumoral growth. This may happen by direct effect³⁵⁻³⁸ or indirectly, by the formation of a local immunosuppressive environment serving as sanctuary against the antineoplastic immune reaction^{35,39-41}.

Therefore, the actual role of TLRs in both physiological and pathological conditions is far more complex than their function as sensors of exogenous threats, and has not been fully understood yet.

TLR signaling

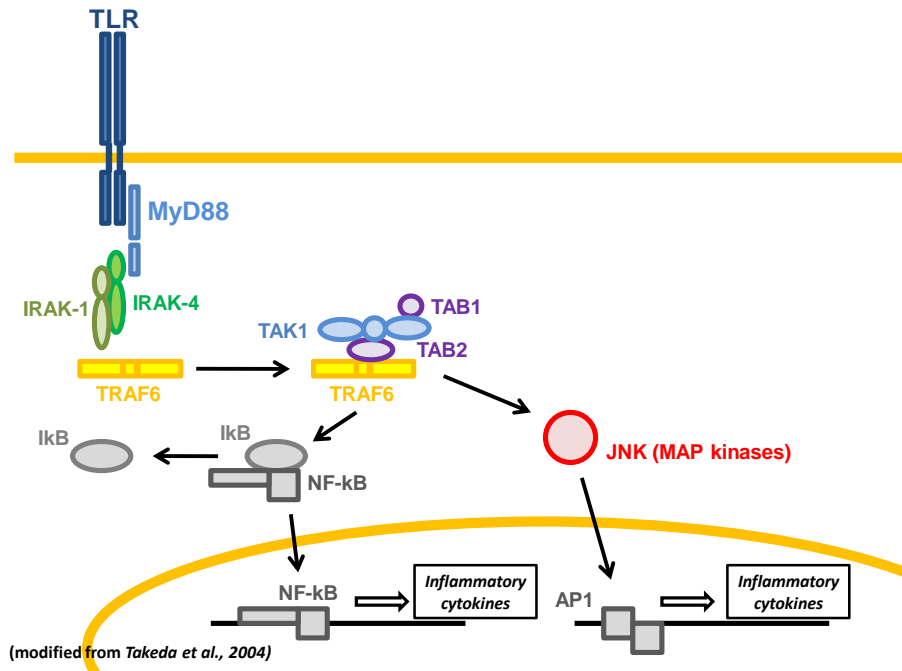
Signaling after TLR activation depends on a cascade of molecular events. As TLRs share with the IL-1 receptor family the intracellular TIR domain, their molecular cascade was expected to be similar, at least in the first phase. In fact, the interaction of MyD88 with TLRs (or IL-1R) accounts for the initiating event in both cases.

MyD88 is a coupling protein characterized by a TIR domain in the C-terminal portion, and a death domain in the N-terminal portion^{4,8}. Through the death-domain of MyD88, TLRs recruit *IL-1 receptor-associated kinases* (IRAKs), which carry the signal forward (**Figure 2**). Of the four members of this family, only IRAK-1 and -4 are the ones catalytically active, while the other two (IRAK-2 and IRAK-M) function as regulators^{11,42}. It was recently shown how IRAK-4 actually acts upstream of IRAK-1⁴³, and, as such, is more important than IRAK-1 in transmitting the signal. Unsurprisingly, IRAK-4 deficiency is associated with a substantial increase in the incidence of pyogenic bacterial infections in humans⁴⁴.

IRAK-4 and IRAK-1, activated by phosphorylation, associate with TRAF6 (*TNF-receptor-associated factor-6*), a common mediator capable of activating both JNK and NF- κ B transcription factors downstream. The IRAK-1/TRAF6 complex physically dissociates from the TLR and interacts at the internal membrane portion with *TGF- β -activated kinase 1* (TAK1) and *TAK1-binding proteins* (TAB1 and TAB2). This large complex then moves in the cytoplasm, where it interacts with other proteins, such as the E2 ligases Ubc13 and Uev1A, that mediate the interaction between TRAF6 and TAK1, and finally the activation of NF- κ B by phosphorylation of its I κ B inhibitory fraction. Alternatively, the TRAF6/TAK1 complex can activate the AP-1 transcription factor by the MAP-kinase signaling proteing JNK (**Figure 2**)¹¹.

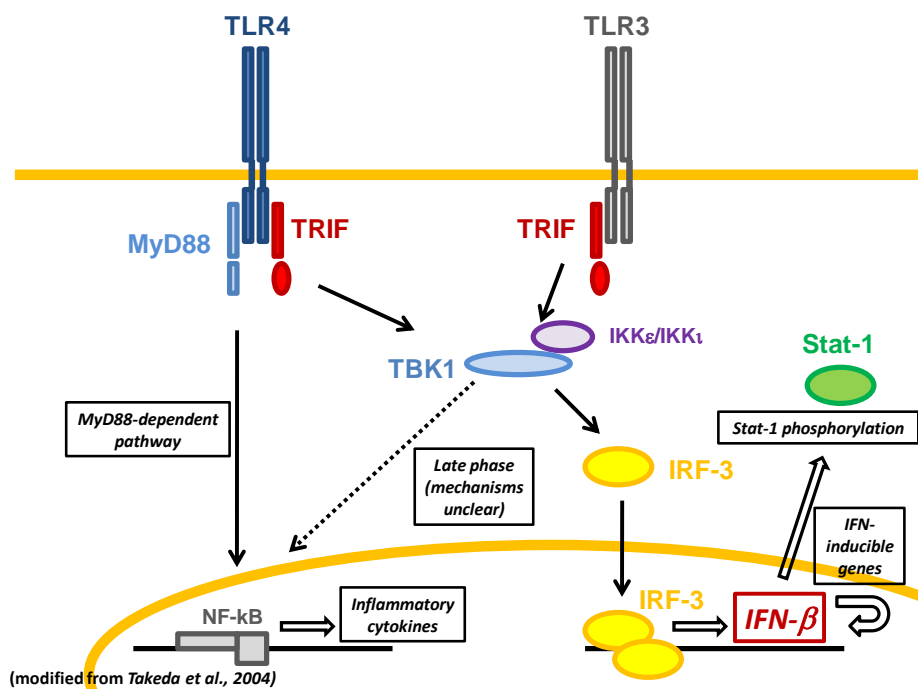
While the MyD88-dependent signaling pathway was first discovered as common to all TLRs except TLR3, an alternative MyD88-independent pathway was identified later as exclusive to TLR3, and, to a lesser extent, TLR4⁴⁵ (**Figure 3**). It appeared crucial in determining the production of interferon- β (IFN- β) in several cell types in response to both natural and synthetic double-stranded RNAs⁴⁶⁻⁴⁷. As a matter of fact, much before TLR3 was discovered, its principal ligand, synthetic poly(I:C), had already been identified as a very powerful inducer of IFN- β ⁴⁷. Following TLRs characterization, the existence of an alternative signaling pathway independent from MyD88 was hypothesized after experiments with the mouse mutant strains C3H/HeJ. These mutants are characterized by a point mutation in the gene codifying the TIR domain that results in the substitution of Proline with Histidine in position 712 of the protein. As such, their TLRs lack the interaction with MyD88 and its downstream signaling pathway⁴⁸. Nonetheless, also in these mutants it was possible to observe interferon production after TLR3 triggering, and there still was a partial, delayed activation of NF- κ B and JNK

FIGURE 2. TLR-mediated MyD88-dependent signaling pathway.



Upon stimulation, IRAK-4, IRAK-1 and TRAF6 are recruited to the receptor. Activated TRAF6 then interacts with TAK1, TAB1 and TAB2 to form a cytoplasmic signaling complex. Phosphorylated TAK1 further activates NF- κ B and AP1 transcription factors *via* IKK complex (*not shown*) and JNK (MAP kinases), respectively.

FIGURE 3. TLR-mediated MyD88-dependent signaling pathway.



Exclusive to TLR3 and TLR4 signaling, the MyD88-independent signaling pathway leads to activation of IRF-3 *via* TRIF, TBK1 and IKK ϵ /IKK ι . The subsequent IFN- β production lead to phosphorylation of Stat-1 and several IFN-inducible genes.

in monocytes after challenging with LPS (the main TLR4-ligand).

These observations were later confirmed by studies in MyD88 knockout mice. In both cases, none of the pro-inflammatory cytokines typically produced in wild-type mice after TLR activation was observed. On the contrary, MyD88 independent pathway activated the *Interferon-Regulatory Factor* (IRF)-3 and by that acted on IFN- β production, resulting in the phosphorylation of Stat1 by interferon, and ultimately in the induction of several IFN-inducible genes, such as IP-10 and GARG16^{45,49-52} (**Figure 3**). This was evident after TLR3 stimulation in both wild-type and MyD88 knockout mice, while it was observed after TLR4 triggering only in MyD88 knockout cells^{50,52}.

In fact, the effective importance of this pathway in determining the effects of TLR4 triggering in physiological conditions is still unclear: for instance, TLR4 stimulation does not result in IFN- β production *via* mobilization of the MyD88-independent signaling pathway in human neutrophils⁵³. Therefore, there may be significant differences at this regard between humans and mice, and depending on the cell types under investigation.

IRF-3 activation in the MyD88-independent pathway follows interaction of TLR3 and TLR4 with *TIR-domain adaptor inducing IFN- β* (TRIF), which serves as alternative coupling protein in the absence of MyD88⁵⁴ (**Figure 3**). In fact, all TLR signaling can be abolished by two mutations (in genes encoding MyD88 and TRIF)². TRIF, by interacting with IKK ϵ and IKK ι , can activate TBK1 and, in turn, NF- κ B, thus explaining the modest level of NF- κ B activation that was observed in early experiments with MyD88 knockout cells challenged with LPS^{11,54}. In any case, these two pathways appear quite distinct, as the role their activation plays in *in vivo* situation: while TLR3 activation by double-stranded RNA ultimately results in IFN- β production and the development of an anti-viral response by MyD88-independent signaling, all the other

TLRs, mainly responsive to extracellular pathogens and bacteria, result in the outburst of proinflammatory cytokines driving the early phase of the inflammatory response.

Signaling by TLRs has several internal modulators: for instance, an alternatively spliced variant of MyD88, lacking the intermediate domain and inhibiting LPS-induced NF- κ B activation, is produced in response to prolonged LPS stimulation⁴³. An additional TIR-domain containing adaptor, TIRAP/Mal, has been shown to mediate the interaction between MyD88 and IRAKs. The role of other molecules, such as *Toll-interacting protein* (Tollip) and members of the *Pellino* family, is still disputed¹¹. Finally, TRIF knockout mice showed a defective TLR4-mediated production of inflammatory cytokines, even though the MyD88-dependent pathway was unaffected by the mutation. This led to the speculation that TLR4 pathway actually requires activation of both MyD88-dependent and -independent pathways to function properly in physiological conditions¹¹. Interestingly, TLR4 activated by lipid A in the absence of CD14 triggers the MyD88-dependent pathway with no influence on the alternative TRIF pathway¹¹.

TLR4 functions in human neutrophils

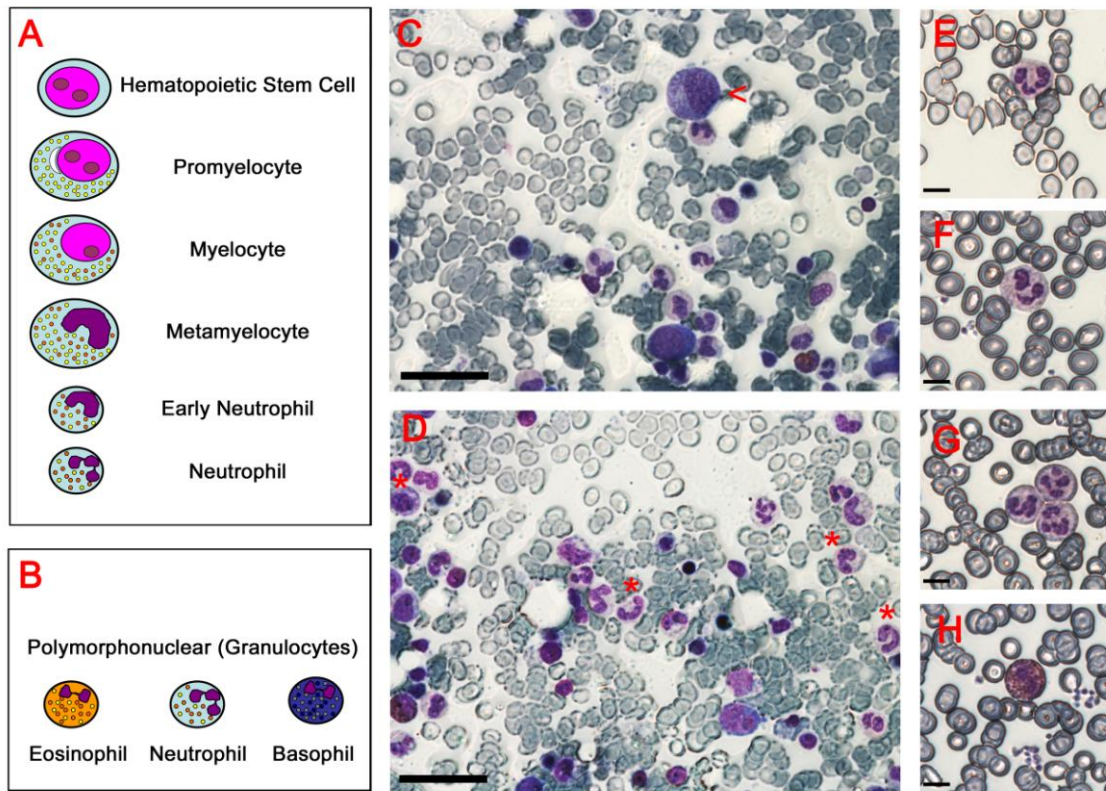
Neutrophils play an essential role in the response to infections by the innate immune system (**Figure 4**). They represent almost two thirds of circulating leukocytes and they rapidly react to the threat posed by an exogenous infection, or tissue damage, by migrating to the site of damage, by limiting infection and by recruiting other immune cells through the release of a wide variety of inflammatory cytokines, chemotactic molecules and antimicrobial products⁵⁵. In most cases, this results in pathogen clearance and/or the initiation of the adaptive immune reaction⁵⁵⁻⁵⁶. Excessive or inappropriate

neutrophil activation, on the other hand, is a pathogenetic step in most autoimmune diseases, such as rheumatoid arthritis, but also inflammatory bowel disease, asthma, chronic obstructive pulmonary disease, all characterized by an on-going excessive inflammation, and acute respiratory distress syndrome, the pulmonary equivalent of systemic sepsis^{55,57-58}.

Human neutrophils express mRNA for all the TLRs, except TLR3^{1,3,53}. They react to the specific ligands of all TLRs, except poly(I:C) (the typical TLR3-ligand)³. CpG DNA (a powerful TLR9 trigger) requires the pretreatment of neutrophils with GM-CSF to elicit a response³. In line with the objects of the present work, we will concentrate on neutrophil activation by LPS, the specific TLR4-ligand⁵⁹.

Neutrophil activation by TLRs involve almost exclusively the MyD88-dependent pathway, and a large amount of data are available from the literature on the possibility to activate NF- κ B⁶⁰ and MAPK signaling⁶¹ following challenge with LPS. The effective role played by the MyD88-independent pathway in neutrophils is still debated, and probably not so significant as previously thought: although LPS can elicit *via* TLR4 the induction of several antiviral genes in neutrophils, and the mobilization of MyD88 independent signaling⁶², experiments performed with highly purified preparations, devoid of monocytes, seem to indicate that this mechanism does not involve the production of IFN- β ⁵³, nor the autocrine IFN- β activating loop observed e.g. in monocytes^{53,62}. Further complicating the picture, the response elicited on JNK activation by TLR4 triggering may be different depending on the fact that neutrophils are kept in suspension or in adherence to a substrate⁶³; how much this influences neutrophil response in an *in vivo* context is still debated, but JNK activation is necessary in neutrophil to release the chemokine monocyte chemoattractant protein-1, important for monocyte recruitment, and may therefore have a role in the cross-talk between

FIGURE 4. Origin and differentiation of polymorphonuclear neutrophils.



A. schematic differentiation of a PMN neutrophil from a Hematopoietic Stem Cell. **B.** Schematic representation of the three types of PMN (granulocytes), as they appear after May-Grunwald-GIEMSA staining. **C-D:** morphological bone marrow smears after May-Grunwald-GIEMSA staining: the arrow indicates a promyelocyte, while asterisks indicate early neutrophils. Various neutrophils at complete differentiation are seen in the field. **E-G:** fully-differentiated neutrophils in a morphological smear of peripheral blood. **H:** a mature eosinophil from the same smear. Magnification: **C-D:** 200x (scale bar = 80 μm); **E-H:** 300x (scale bar = 10 μm).

these cell types during inflammation⁶³.

TLR triggering by PAMPs induces responses in a variety of cells *in vivo*, primarily epithelial cells, endothelial cells, and monocytes/macrophages. Activation of these cells by TLRs may indirectly drive neutrophil migration, e.g. by modulating the expression of endothelial adhesion molecules. At the same time, it may result in the selective recruitment of neutrophils in specific danger zones, e.g. by cytokine and chemokine production¹.

TLRs modulate neutrophil migration and activation by many different mechanisms. First, an indirect mechanism has been proposed, by which TLR4 triggering results in the down-regulation of G-protein receptor kinase, responsible for the desensitization of CXCRs to their ligands, and therefore determines an enhanced sensitivity of neutrophils to chemotactic stimuli⁶⁴. Then, TLR4 engagement by LPS results in delayed neutrophil apoptosis^{3,65-66}, increased L-selectin shedding and the upregulation of several adhesion molecules, e.g. CD11b⁶⁶. Finally, many inflammatory mediators, present at sites of infection, regulate neutrophil chemokine receptors and, therefore, their additional chemiotaxis.

While enhancing migration ability, TLRs also play a pivotal role in the establishment of a local inflammatory infiltrate. In fact, once localized, neutrophils lose much of their migration ability: LPS-activated neutrophils progressively lose IL-8-binding capacity, as well as down-regulate IL-8Rs^{3,67} and CXC chemokine receptor (CXCR)-1 and -2⁶⁸. Among these, CXCR2 is known to play a pivotal role in neutrophil recruitment, and to be directly downmodulated after TLR4 stimulation¹. These combined actions help stabilize neutrophils where they are needed to exert an effective defense.

Once localized, neutrophils act against pathogens by phagocytosis and by the generation of large amounts of reactive oxygen intermediates, cytokines and chemokines⁶⁹. Neutrophil sensitivity to local pathogens, as well as many of the features of activated neutrophils, depend on TLRs^{3,9-10,62}: among them, LPS seems to have a pivotal role in priming neutrophils to enhanced respiratory burst ability^{3,66}, but TLR-stimulated neutrophils have also shown increased phagocytosis of opsonized targets³. TLR2 is also a crucial neutrophil activator, without the strict requirements of serum (i.e. LPS binding proteins) or CD14 to elicit its own response¹. Studies with selective agonists have tried to dissect TLR4- from TLR2-dependent effects; these have been further complicated, until recently, by the technical difficulties existing in obtaining highly purified neutrophil preparations, as well as by the frequent presence of traces of contaminants in commercially-available LPS batches¹. In experiments that took these issues into account, TLR2 and TLR4 appeared to induce a very similar activated state in neutrophils. To date, the only significant difference between the two has been observed with regard to survival and the generation of reactive oxygen intermediates, which both appear more effectively increased by TLR4 triggering than TLR2^{66,70}; the difference, however, is merely quantitative. Interesting enough, both these studies confirmed the direct role of LPS in arresting early apoptosis (4h), by activation of the MyD88-dependent pathway; they did not confirm, though, earlier observations that indicated a prolonged neutrophil survival, up to 22h, following LPS stimulation. Instead, this was explained by the presence of contaminating monocytes in earlier studies^{66,70}. Thus, it is currently thought that TLR4 activation on neutrophils by LPS alone results in a direct, yet temporally-limited, delay of spontaneous apoptosis¹.

In summary, TLR4 activation by LPS is the first step in a complex network of alterations that ultimately powerfully affect neutrophil viability, trafficking, migration,

adhesion to inflamed endothelia and function as tissue infiltrate. All these actions cooperate in providing a powerful inflammatory response against infections.

MESENCHYMAL STROMAL CELLS

Definition and characterization

Multipotent mesenchymal stromal cells (MSC), formerly known as *mesenchymal stem cells*, are the precursors of tissue stromal cells and fundamental elements of tissue homeostasis⁷¹⁻⁷³. Originally isolated from the bone marrow (BM) as nurse cells for the committed hematopoietic lineages and part of the hematopoietic niche⁷⁴⁻⁷⁵, MSC can be expanded from virtually all tissues⁷⁶, including peripheral blood⁷⁷, and thus form a complex stromal system throughout the body. Although the real *in vivo* counterpart of culture-expanded MSC still remains a matter of debate, after a decade of intense research much is known about their *in vitro* characteristics and the protocols to isolate and successfully expand them⁷⁸. According to the definition criteria adopted by *The International Society for Cellular Therapy (ISCT)* MSC have to show a homogeneous immunophenotype after short *in vitro* culture (passage 2-3); they express a minimal panel of mesenchymal markers (e.g. CD106, CD90, CD44, CD29), without evidence of hematopoietic (e.g. CD34, CD45, CD117/c-kit) or endothelial (e.g. CD31, vWF) differentiation; they undergo clear multipotent differentiation in at least three differentiated progenies of the same embryonal layer (mostly adipocytes, osteoblasts and chondrocytes) after exposure to specific induction media⁷⁹. International consortia have been formed to standardize the procedures for cell collection, culture and tests prior to clinical use in large international trials of regenerative medicine⁸⁰.

Among other functions, MSC display powerful immune modulatory properties towards the main immune effector cells⁸¹⁻⁸³. The activation of T-lymphocytes by either TCR-dependent⁸⁴ or unspecific stimuli⁸⁵ is prevented by BM-MSC under *in vitro* coculture conditions; such effects are mediated by redundant mechanisms depending on both contact-dependent interactions⁸⁴ and soluble factors⁸³⁻⁸⁴, and their effect is pleiotropic. MSC also positively or negatively affect B-cell proliferation, according to their priming by inflammatory cytokines⁸⁶⁻⁸⁸, and they may inhibit NK^{81,89} and dendritic cell reactivity towards allogeneic cells^{81,90}. In addition, they significantly interact with monocytes and macrophages *in vitro* and *in vivo*; their administration in a mouse model of enterogenic sepsis has lowered inflammation and improved survival through the prostaglandin E₂-dependent modulation of monocytic IL-10 production⁹¹.

Overall, MSC and their progeny⁹² seem to act as a competitive system that prevents excessive reactions towards pathogens, thus contributing to the resolution of immune responses⁸²⁻⁸³. MSC immune modulatory properties have been demonstrated also *in vivo*⁹³, where they appear to be finely tuned by the local microenvironment⁸²⁻⁸³. For instance, inflammatory cytokines, such as IFN- γ or TNF- α , “prime” MSC for enhanced suppressive mechanisms⁹⁴, while infectious agents may hamper MSC inhibitory effects towards lymphocytes and other cell types through the engagement of Toll-like receptors (TLRs), such as TLR3 and TLR4²¹.

Role of TLR3 and TLR4 on Mesenchymal Stromal Cells

Expression of mRNAs for TLRs in BM-MSC has been assessed by many different studies²¹⁻²³: of the ten receptors known in humans, only TLR2, -3 and -4²¹⁻²² have proved to be expressed at high level (as mRNA), and to be functionally present as proteins on the surface of MSC²¹⁻²³. TLR1, TLR5, TLR6, on the other hand, are

expressed at low level as mRNA only^{21-22,95}. TLR9 was detected as mRNA, but not as functional protein, in adipose-tissue derived MSC⁹⁵, while, by contrast, a more recent publication reported constitutive expression of TLR9 by resting human BM-MSc, and increased tissue invasiveness in experimental conditions after exposure to its corresponding ligand, CpG DNA⁹⁶.

The role of TLR2, -3 and -4 in MSC is rather different: the first one, upon stimulation, maintains BM-MSc in a proliferative and self-renewing state without affecting neither their immunosuppressive properties⁹⁷ nor their potential for multipotent differentiation⁹⁷. On the contrary, TLR3 and -4 do not appear to predominantly affect differentiation²¹, but rather to influence BM-MSc stress responses and migration²². Most of all, they are pivotal elements in the regulation of the powerful immunomodulative effects exerted by BM-MSc.

Initial findings seemed to indicate that the engagement of TLR3 and TLR4 on MSC could dynamically revert the baseline immunomodulatory functions exerted by these cells, enabling a normal response of lymphocytes to either specific and unspecific stimuli even in the presence of MSC in coculture²¹. The mechanism underlying this function consists in a TLR3/4 dependent down-regulation of MSC surface Jagged1. Besides acting on immunomodulatory functions, the triggering of TLR3 and TLR4 also convert BM-MSc into powerfully chemotactic cells by increasing their production of IL-1 β , IL-6, IL-8/CXCL8, CCL5, IP10/CXCL10 and monocyte chemotactic protein (MCP)-1 *via* activation of NF- κ B signaling^{21-23,98}. Similar results have been obtained in adipose-tissue derived MSC, where TLR agonists increased mRNA production of MCP-1 and -2, granulocyte chemotactic protein-2 (GCP-2), IL-1 β , macrophage inflammatory protein-3 α (MIP-3 α), TNF- α and IL-12⁹⁵. Both MyD88-dependent and MyD88-

independent pathways have shown to be involved in transmitting the signals downstream of TLR4 activation on MSC⁹⁹.

As such, TLR3 and TLR4 stimulation on MSC was initially thought to induce a dynamic, reversible block of their immunosuppressive features and to switch these cells towards the production of several chemokines and proinflammatory cytokines, thus helping the proper development of inflammation in the presence of exogenous threats, such as bacteria (sensed by TLR4) and viruses (TLR3)⁹⁸.

Later studies proved the situation to be rather more complicated. First of all, the functional, dynamic block of MSC immunomodulative properties by TLR3 and TLR4 stimulation reported by Liotta and colleagues required IDO1 activity to be bypassed¹⁰⁰. Then, effects of TLR triggering on MSC seemed to depend on tissue source of expanded cells, as TLR2, TLR3 and TLR4 ligation did not appear to affect the ability of human adipose tissue-derived MSC to suppress lymphocyte activation¹⁰¹. The condition of the microenvironment could also influence the response to TLR triggering: e.g. culturing MSC in an inflammatory milieu resulted in the upregulation of TLR2, TLR3 and TLR4, and in an increased proinflammatory shift in their cytokine profile after exposure to both poly(I:C) and LPS¹⁰². Finally, conflicting results were recently described in lymphocyte:BM-MSC cocultures after short-term (1 hour), low-level (poly(I:C) 1 µg/ml; LPS 10 ng/ml) stimulation of BM-MSC with either TLR3- or TLR4-ligands¹⁰³. In this latter study, TLR4-primed MSC exhibited a pro-inflammatory profile, with increased levels of cytokines such as IL-6, IL-8/CXCL-8 or TGF-β, while TLR3-primed MSC exerted increased immunosuppressive activities, by producing mainly IL-10, IDO and PGE2¹⁰³. Interestingly, multipotent differentiation and extracellular matrix deposition in this model were also differently affected after TLR3

or TLR4 activation¹⁰³. In analogy with the M1/M2 monocyte/macrophage polarization¹⁰⁴, a similar functional priming has therefore been hypothesized for MSC by these authors¹⁰³: accordingly, a *MSC1* “pro-inflammatory” would oppose to a *MSC2* “immunosuppressive” phenotype¹⁰³, and the specific activation of different TLRs on MSC would push the cells towards one phenotype or the other.

However, there is evidence that TLR3 or TLR4 activation on MSC results in very different outcomes depending on the specific experimental conditions, on the concentration of the respective ligands, on the duration of the stimuli and on the coculture ratios with interacting cells. As shown by others^{95,101,105}, the pattern of expression of TLRs and the outcome of their signaling may also depend on the tissue source used to expand MSC, even though BM-MSCs still serve as the fundamental model for all other types of MSC.

Therefore, the real capacity of TLR4 to polarize MSC specifically towards *MSC1* phenotype, and, on the other hand, the ability of TLR3 to promote an exclusive *MSC2* polarization, as proposed by Waterman and colleagues¹⁰³, is still uncertain.

Do Mesenchymal Stromal Cells affect Neutrophil biology ?

As it is well known, besides acting as first line phagocytes, neutrophils (PMN) are far more complex cells, capable of intense biological activity⁵⁵. Upon challenge by various stimuli, they release lytic enzymes with powerful antimicrobial potential, and generate reactive oxygen intermediates (ROI), which are essential for pathogen killing⁵⁵. Neutrophils can also be induced to produce *de novo* a variety of mediators involved in their functions⁶⁹ and, in turn, they cross-talk with immune¹⁰⁶⁻¹⁰⁸ and non immune effector cells¹⁰⁹. Nevertheless, it was unclear until recent studies whether an interaction existed between these cells and BM-MSCs.

In 2008, a study by Raffaghello and colleagues clarified that while neutrophils are normally short-living cells, they display a significantly lower tendency to undergo apoptosis when cocultured with untreated BM-MSC, for up to 40 hours and particularly if coincubated at high PMN:BM-MSC ratio (50:1)¹¹⁰. Concomitantly, the capacity of neutrophils of producing hydrogen peroxide upon fMLF-stimulation decreases under coculture conditions, while their chemotactic ability or their CD11b or CD62L expression remain unaffected¹¹⁰. All these effects are not only reproduced by culturing neutrophils in BM-MSC-conditioned supernatants, but also fully dependent on IL-6¹¹⁰, which is constitutively produced at high concentrations by BM-MSC^{75,110}.

More recently, supernatants from parotid-derived MSC stimulated *via* TLR4 by LPS also proved to significantly improve neutrophil survival and chemotaxis¹¹¹. However, even if MSC supernatants were shown in this study to contain G-CSF, IL-6, IL-8/CXCL8 and MIF, no functional analysis was performed to identify the factors specifically responsible for their effects¹¹¹.

It is currently unknown whether other factors could influence the relationship between neutrophils and BM-MSC. At the same time, the mechanisms by which these cells interact are still unclear, even if soluble factors are thought to be responsible for the observed effects.

AIM OF THE STUDY

In the present study, we examined whether MSC isolated and expanded *in vitro* from bone marrow, thymus, spleen and adipose tissue can modify neutrophil survival, phenotype and function after activation by either poly(I:C), a specific ligand for TLR3, or LPS, a ligand for TLR4.

We demonstrate here that both TLR3 and TLR4 activation result in significantly increased and prolonged survival by neutrophils, much more potent than what observed in resting conditions. At the same time, we demonstrate this effect to be linked to neutrophil activation, as showed by increased CD11b expression and enhanced potential for respiratory burst activity after coculture. We show that TLR3-activated BM-MSC are more powerful than TLR4-stimulated BM-MSC in exerting such effects, and that these properties are shared by all the types of MSC that we used, regardless of their source (bone marrow, thymus, spleen and adipose tissue).

Finally, we test the mechanisms for these effects, by virtually reproducing all results with supernatants taken from TLR3- and TLR4-stimulated BM-MSC, by testing their content of cytokines well-known for their action on neutrophil survival and functions (IL-6, IL-8, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, TNF- α), and by performing blocking experiments by means of specific neutralizing monoclonal antibodies. We show that a concerted action of endogenously produced IL-6, IFN- β and GM-CSF determines most of the modulatory effects exerted on neutrophils by TLR3-activated BM-MSC, while GM-CSF is solely responsible for most of those exerted by TLR4-activated BM-MSC.

Taken together, these observations highlight a novel mechanism by which tissue resident MSC may sustain and amplify the functions of neutrophils upon TLR activation in physiological and pathological conditions.

MATERIALS AND METHODS

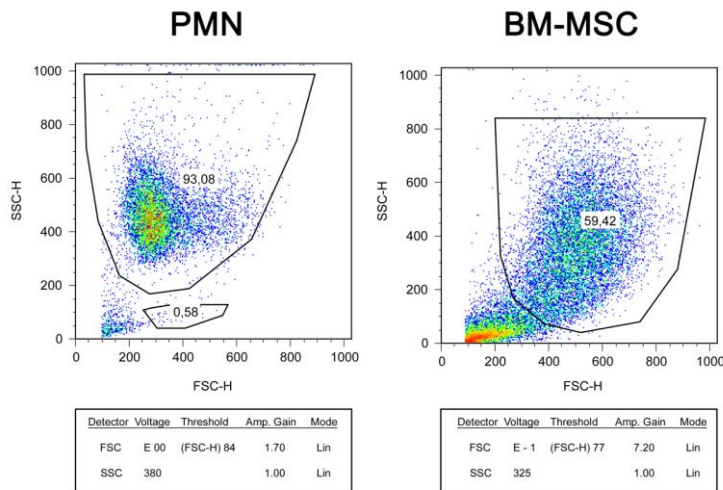
Neutrophil purification

Following isolation under endotoxin-free conditions from buffy coats of healthy blood donors, as described elsewhere^{53,107,112}, neutrophils were enriched by immunomagnetic depletion of committed blood cells, which was obtained using a customized EasySep® kit (Stem Cell Technologies/Voden, Casorezzo, Italy), consisting of a mixture of monoclonal antibodies against human CD3, CD19, CD36, CD49d, CD56 and glycoprotein-A^{53,107,112}. PMN were then resuspended in endotoxin-free RPMI supplemented by 10% FBS (EU LPS level) and 1% penicillin/streptomycin (all purchased by GIBCO/Invitrogen, S.Giuliano Milanese, Italy), and used immediately. Purity was tested by monoclonal antibodies against human CD16, CCR3, CD3 (all by BD Biosciences, Buccinasco, Italy), CD66b (Beckman-Coulter, Milano, Italy), and flow cytometry (BD FACScalibur™) (**Figures 5-6**). Mean final purity was 99.1% (range: 98.5 – 99.8%).

Mesenchymal Stromal Cells purification and culture

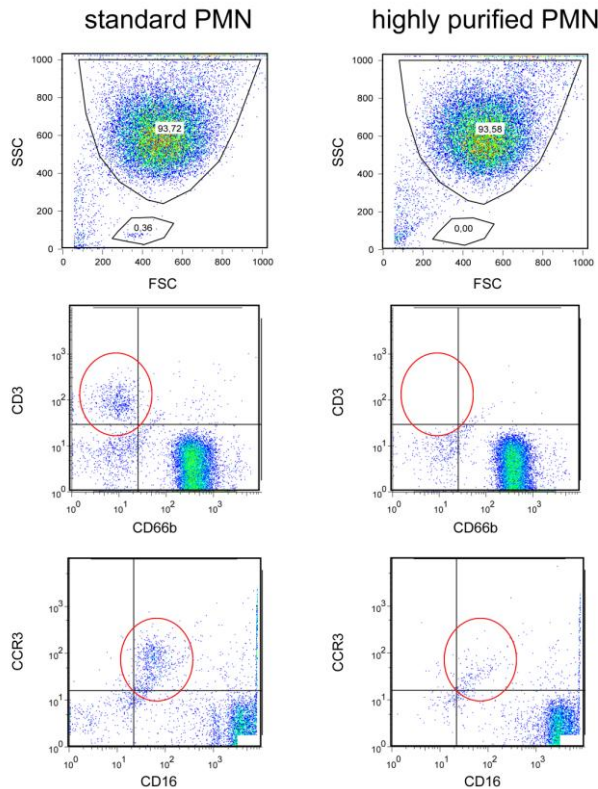
Mesenchymal stromal cells (MSC) were isolated from bone marrow (BM), thymus, spleen and subcutaneous lipoaspirates obtained from healthy donors after informed consent and expanded *in vitro*. Briefly, following density gradient centrifugation, BM mononuclear cells were plated without sorting at the initial (P0) density of 1.2×10^6 cells/cm² in DMEM supplemented with 18% FBS and 1% penicillin/streptomycin (*complete medium*; all by GIBCO/Invitrogen, S. Giuliano Milanese, Italy). To obtain thymic, splenic and adipose-tissue MSC, small fragments of each tissue and 50 ml of lipoaspirate were collected from healthy donors undergoing the

FIGURE 5. Cytofluorimetric physical parameters of Neutrophils and BM-MSc.



Neutrophils are clearly distinguishable from BM-MSc in a morphological cytofluorimetric gate (SSC-H / FSC-H) because of their very different physical parameters.

FIGURE 6. Effect of immunomagnetic purification of Neutrophils.



Standardly isolated Neutrophils have been stained and analyzed by FACS to test their purification level and potential contaminant cells, e.g. CD16^{low}/CCR3⁺ cells (eosinophils) or CD3⁺ cells (T-lymphocytes; both indicated by the red ovals). Analysis is performed on the morphological gate indicated in the first row and based on PMN physical parameters. The second column shows the disappearance of most of the contaminant cells after the immunomagnetic depletion with mAbs targeted against specific markers of blood cells other than neutrophils.

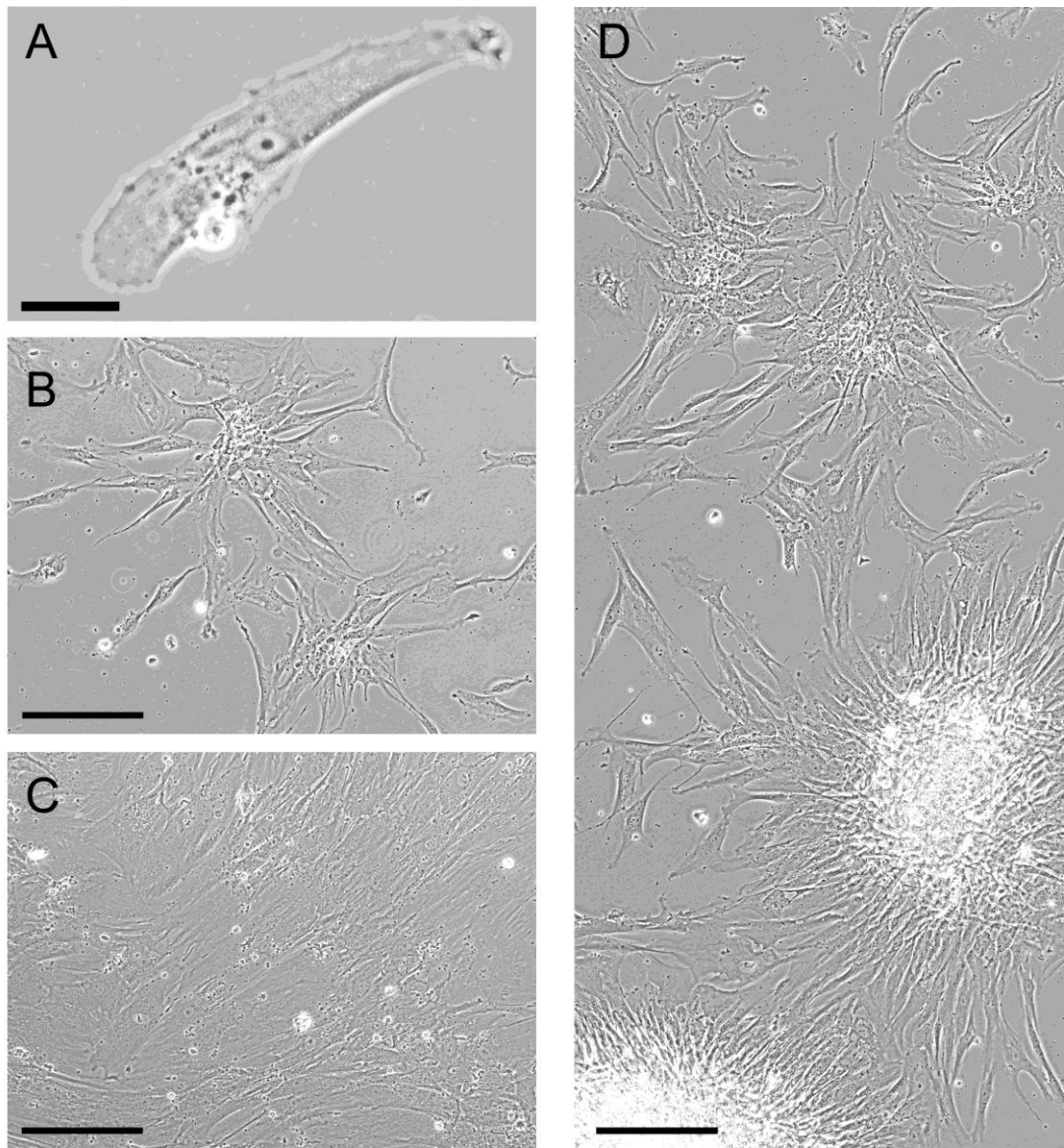
specific surgical procedure for unrelated reasons. Samples were digested with a collagenase solution (0.075% in HBSS) at 37 °C in a thermic steady state shaking incubator (120 rpm) for 30 minutes^{78,113}. The cell suspensions derived from the disaggregation of thymic and splenic samples were then collected, the collagenase was neutralized by dilution in *complete medium* at a 1:3 ratio, the cellular pellet was collected after centrifugation, and contaminating erythrocytes were lysed by means of a 160 mM NH₄Cl solution (Sigma-Aldrich, Milano, Italy) (10 minutes, room temperature). Finally, cells were plated in *complete medium* at the initial density of 10 x 10⁶ cells/cm² for thymic cells and 5 x 10⁶ cells/cm² for splenocytes¹¹³. The adipose tissue-derived cell suspension containing the vascular-stromal fraction was collected after collagenase neutralization and centrifugation at 200g for 10 minutes at room temperature. Cells were then filtered through a 100 µm cell strainer and plated in *complete medium* at the initial density of 1 x 10⁶ cells/cm², as described elsewhere^{78,113}.

Following a brief expansion in *complete medium*, cells displayed a homogeneous mesenchymal immunophenotype starting from passages 2-3 (P2-3) (**Figures 5 and 7**) and proved capable of *in vitro* multilineage differentiation into adipocytes, osteoblasts and chondrocytes after exposure to specific differentiating media, as described elsewhere^{78,113}. Medium was entirely changed with fresh RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at the start of each coculture experiment.

Immunophenotypic analysis was performed after 2 or 3 passages (P2-3) using monoclonal antibodies against human CD106, CD105, CD73, CD80, CD86, CD45, CD34, CD31, CD11c, CD146 (all by BD Biosciences, Buccinasco, Italy) and flow cytometry^{78,113} (**Figure 8**). For each experiment 10⁴ events were counted.

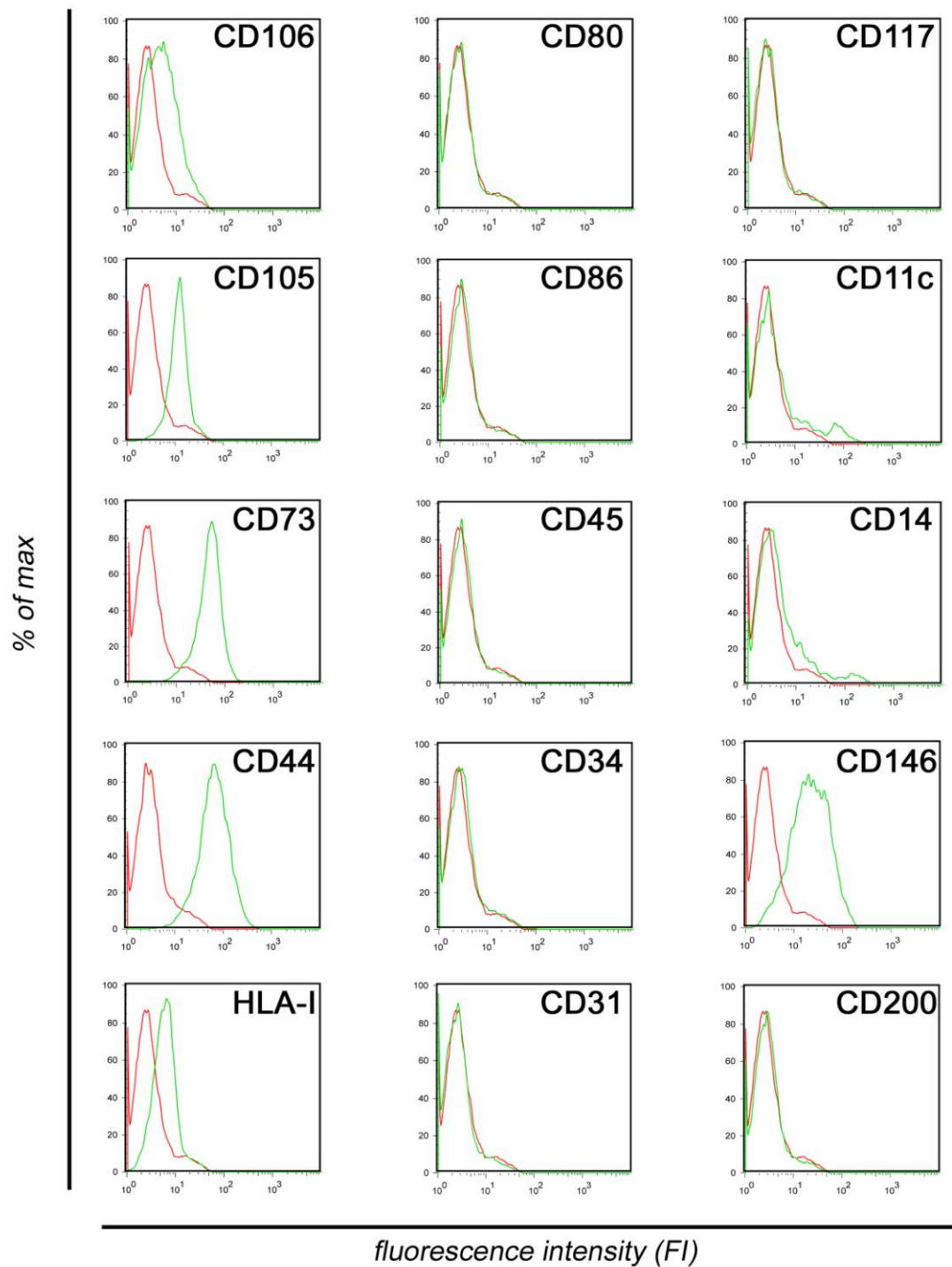
Expanded cells displayed the ability to differentiate *in vitro* into adipocytes, osteoblasts and chondrocytes after exposure to specific induction media,

FIGURE 7. BM-MSC *in vitro* culture.



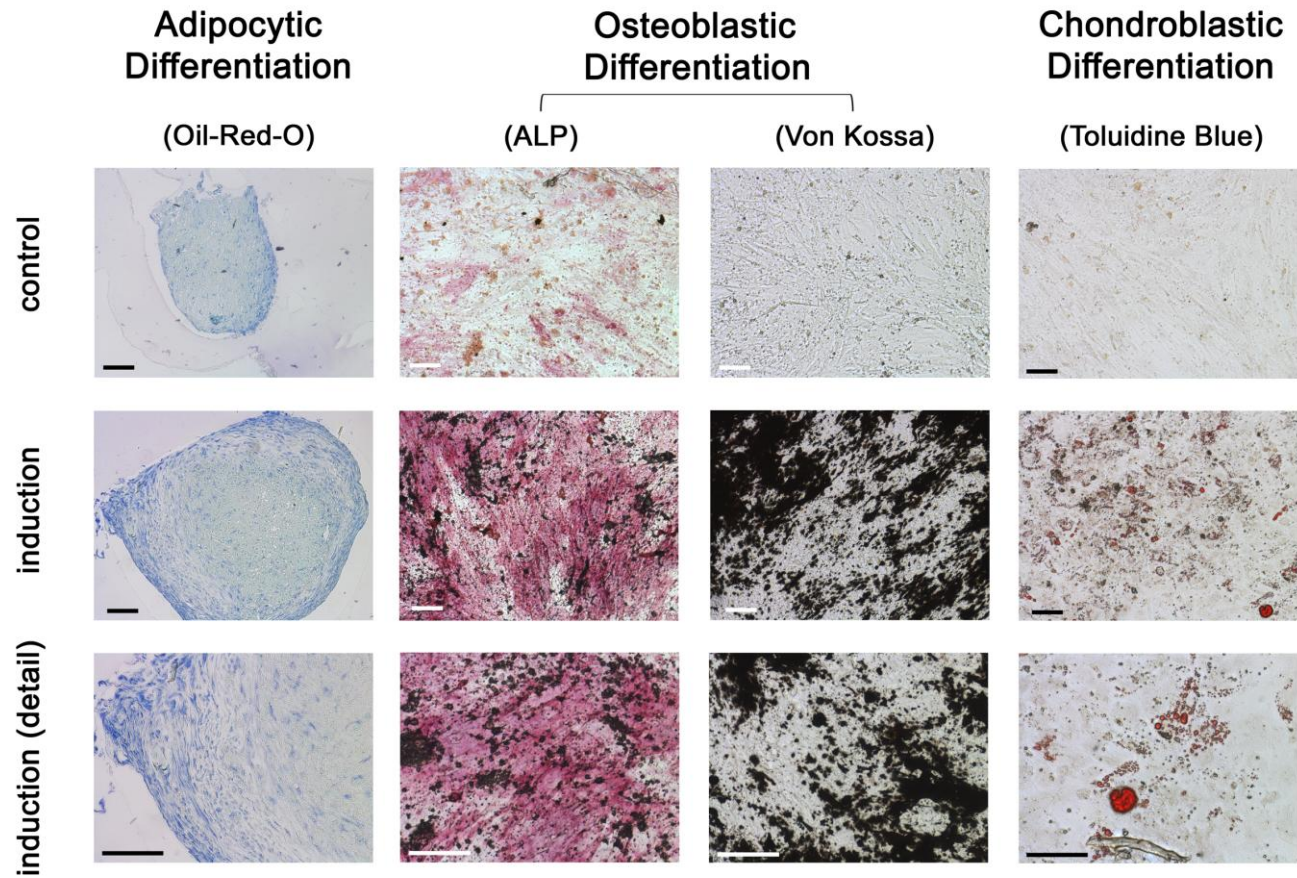
BM-MSC growth *in vitro*. A-C: during expansion, BM-MSC tend to progressively grow from single cells (A) to clusters of spindle-shaped cells (B), to confluent monolayers (C). D: Clusters of cells grow to progressive confluence. Magnification: A: 400x (scale bar = 40 μm. B-D: 100x (scale bar = 200 μm).

FIGURE 8. Immunophenotype of *in vitro* expanded BM-MSC (P4).



The expression of each marker is highlighted in green as frequency distribution of fluorescence intensity, and compared to that of isotype controls (red lines).

FIGURE 9. Multipotent differentiation of BM-MSC *in vitro*.



Columns indicate differentiated lineages obtained after exposing BM-MSC to the respective induction media for 21 days, and revealed by specific histochemical stainings. First row indicates BM-MSC kept in standard culture medium in the same conditions, and used as controls. Magnification: first two rows: 100x (scale bar = 100 μ m); third row (detail): 200x (scale bar = 100 μ m).

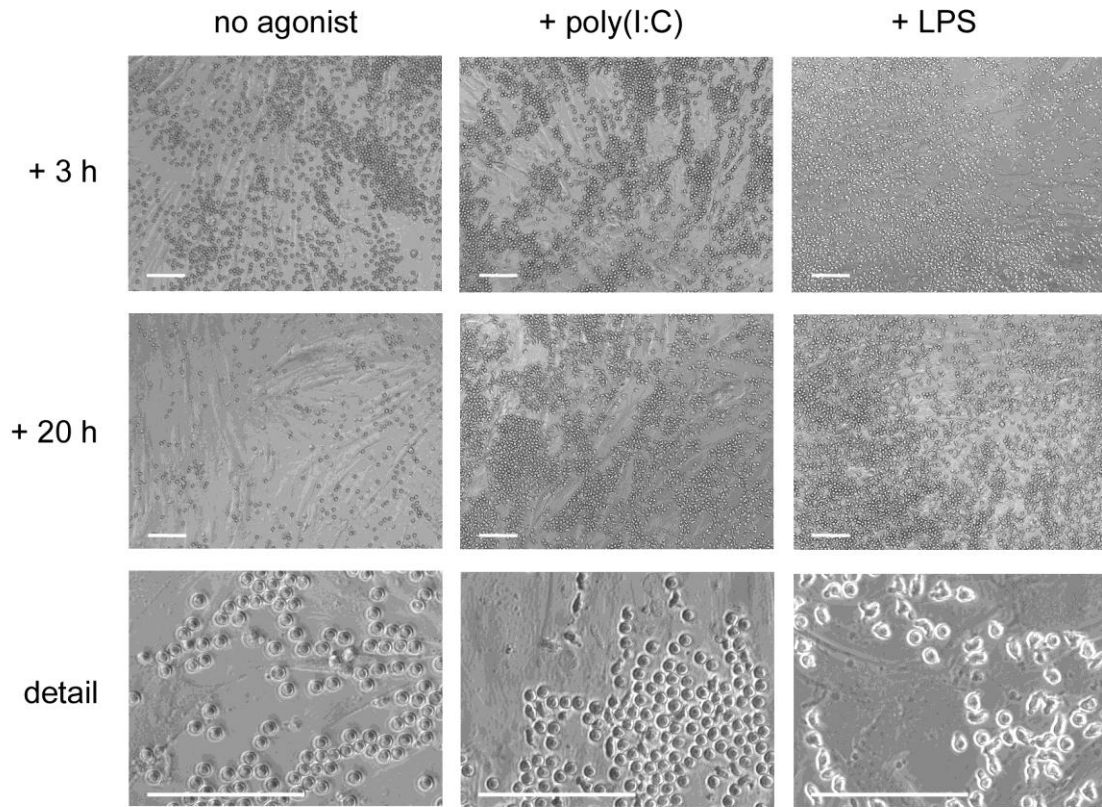
as described elsewhere^{78,113} (**Figure 9**).

PMN:MSC cocultures

Neutrophils (99.1% ± 0.5% purity) and MSC were cocultured for up to 44 hours (**Figure 10**) in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (all by GIBCO/Invitrogen, S.Giuliano Milanese, Italy), at PMN:MSC ratios ranging from 1,000:1 to 10:1, in the presence or absence of 100 µg/ml poly(I:C) (InvivoGen, San Diego, CA) or 100 ng/ml LPS (Ultra-Pure E.coli LPS, Alexis Biochemicals, San Diego, CA), according to preliminary experiments performed to determine the optimal working concentrations (**Figure 11**). In all cases, MSC were plated 24 hours before the start of cocultures. In selected experiments, neutrophils were cultured on top of 0.4 µm-pore size Transwell® inserts (Corning Costar, Cambridge, MA) to prevent cell contact between neutrophils and MSC. At the end of the incubation, neutrophils were harvested by careful pipetting, ultracentrifuged at 600 *x* g for 5 minutes, and finally resuspended in PBS for subsequent assays. Full integrity of MSC layers was checked in all cases.

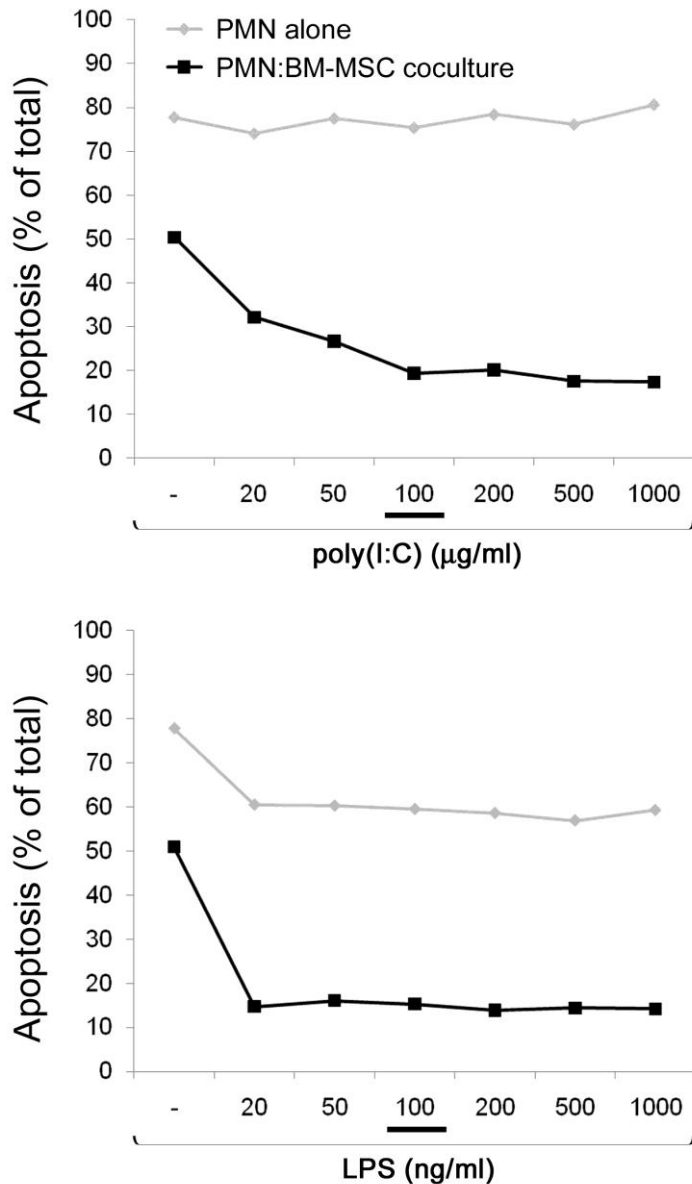
MSC-conditioned media were obtained by incubating the same MSC batches used for the cocultures with neutrophils in the presence or absence of poly(I:C) or LPS for 24 hours, and then by collecting and processing their cell-free supernatants. Cytokine blocking experiments were conducted by culturing neutrophils 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, Minneapolis, MN), IL-8/CXCL8 (PeproTech, Rocky Hill, NJ),

FIGURE 10. Neutrophils : BM-MSC coculture.



BM-MSC have been plated on the bottom of 24-48-96-well plates 24 h before the start of coculture in order to obtain a confluent monolayer. Following fresh isolation, neutrophils have been plated on top of the monolayer or, alternatively, in a Transwell insert, when needed. Culture medium has been changed at the start of the coculture. Magnification: 100x (first and second rows); 400x (third row). Scale bar represents 100 μ m.

FIGURE 11. Modulation of neutrophil apoptosis by BM-MSC after coculture with increasing concentrations of poly(I:C) or LPS.



Neutrophils (PMN) were cultured for 20 hours with or without BM-MSC at 10:1 ratio, in the absence or presence of the indicated concentrations of poly(I:C) or LPS. Apoptosis was measured and neutrophils that were double negative by the Annexin-V/PI method (see *Materials & Methods*) were considered as viable: their percentage is reported as percentage of total. One representative experiment for each TLR-agonist is presented.

IFN- γ (clone B133.3, kindly provided by Dr Giorgio Trinchieri, National Cancer Institute, Frederick, MD) and TNF- α (clone B154.2, kindly provided by Dr Giorgio Trinchieri, National Cancer Institute, Frederick, MD). Alternatively, neutrophils 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), prior to their further culture.

Cytofluorimetric analysis

After isolation from MSC, neutrophils were identified on the basis of their typical morphological parameters (FSC/SSC) (**Figure 5**). We tested the level of neutrophil apoptosis by the Annexin-V-FLUOS Apoptosis Detection kit by Roche Diagnostics (Mannheim, Germany) and flow cytometry analysis (FACS, BD FACScalibur™). Following collection from the well plates, neutrophils were ultracentrifuged at 3000 rpm and the pellet resuspended in 100 μ l of isotonic binding buffer; neutrophils were then incubated at the final concentration of 4×10^3 PMN/ μ l in a working solution of Annexin-V/propidium-iodide (1:100 stock dilution, in isotonic binding buffer), 15 minutes in the dark, additioned with 250 μ l PBS and immediately analyzed by flow cytometry (BD FACScalibur™). The expression of CD16, CD11b and CD64 on neutrophils was also evaluated by FACS (all antibodies by BD Biosciences, Buccinasco, Italy; incubation for 30 minutes at 4 °C; 10^4 events counted for each condition). Prior to the staining with the specific fluorochrome-conjugated antibody, neutrophils were resuspended and incubated with human serum (10% solution) for 30 minutes at 4 °C.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokine concentrations in BM-MSC-free supernatants were measured by commercially available ELISA kits for: human IL-6 (*detection limit 0.6 pg/ml*; Immunotools, Friesoythe, Germany), IL-8/CXCL8 (*detection limit 1 pg/ml*; Immunotools, Friesoythe, Germany), 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, Friesoythe, Germany), G-CSF (*detection limit 0.4 pg/ml*; R&D Systems, Minneapolis, MN), GM-CSF (*detection limit 2 pg/ml*; BioLegend, San Diego, CA), TNF- α (*detection limit 0.09 pg/ml*; human high-sensitivity kit by Invitrogen, Cat.# KHC3014, S.Giuliano Milanese, Italy), according to the respective manufacturer's instructions.

Respiratory burst

Superoxide anion (O_2^-) release was estimated by the cytochrome C reduction assay¹¹⁴. Briefly, following neutrophil/BM-MSC coculture in a 96-well plate, medium was gently removed and replaced 0.2 ml of HBSS/well (pH 7.4), containing 80 μ M cytochrome C (Sigma-Aldrich, Milano, Italy) and 2 mM NaN_3 , in the presence or absence of 100 nM N-formylmethionyl-leucyl-phenylalanine (fMLF) (Sigma-Aldrich, Milano, Italy). The plate was then analyzed by an automated plate-reader (ELX808, Bio-Tek Instruments, Winooski, VT), prewarmed at 37 °C, and absorbance at 550 nm and 468 nm was recorded every 5 minutes. O_2^- production was calculated in nanomoles/ 1.5×10^5 PMN/min using $24.5 \text{ mM}^{-1} \text{ cm}^{-1}$ as extinction coefficient. Each condition was performed in groups of triplicate samples.

Western blot analysis and native gels

After stimulation with LPS or poly(I:C), 1×10^6 BM-MSC for each condition were diluted in ice-cold D-PBS and centrifuged twice at 300g for 5 minutes at 4 °C. The resulting cell pellets were lysed with radioimmune precipitation assay (RIPA) buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) containing 1 mM DTT and antiprotease and antiphosphatase mixtures. Following a 20-minute incubation on ice, samples were centrifuged at 13000g (15 minutes, 4 °C), and the resulting supernatants were divided in aliquots and immediately stored at -80 °C. Small aliquots of the various samples were routinely processed for protein content determination by using a protein assay kit (Bio-Rad, Segrate, Italy). For Western blot analysis, protein extracts were subjected to electrophoretic separation on SDS-PAGE and subsequent transfer to nitrocellulose by electroblotting using standard procedures. Nitrocellulose membranes were first blocked for 1 hour at room temperature in Odyssey buffer and then incubated overnight at 4 °C, in the presence of specific primary antibodies in the same buffer. Antibodies against phospho-tyrosine STAT1, phospho-IRF3, phospho-p38 and phospho-ERK were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies against total-STAT1, IRF3 and I κ B α were purchased from SantaCruz (Santa Cruz, CA), while antibodies against β -actin were purchased from Sigma-Aldrich. Detection was carried out with Alexa Fluor 680 goat anti-rabbit Ab (Molecular Probes/Invitrogen, S.Giuliano Milanese, Italy) and IRDye TM800-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) secondary Ab. Blotted proteins were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK). Native PAGE was performed as previously described¹¹².

Statistical analysis

One-way Analysis of Variance (ANOVA) and Holm-Sidak test for multiple comparisons were used to statistically evaluate the difference of sample means among multiple groups. A *P* value <0.05 was considered as significant. Data are represented using mean \pm standard deviations (SD) in all cases. Calculations have been performed using STATA IC v.10.0 (StataCorp, College Station, TX).

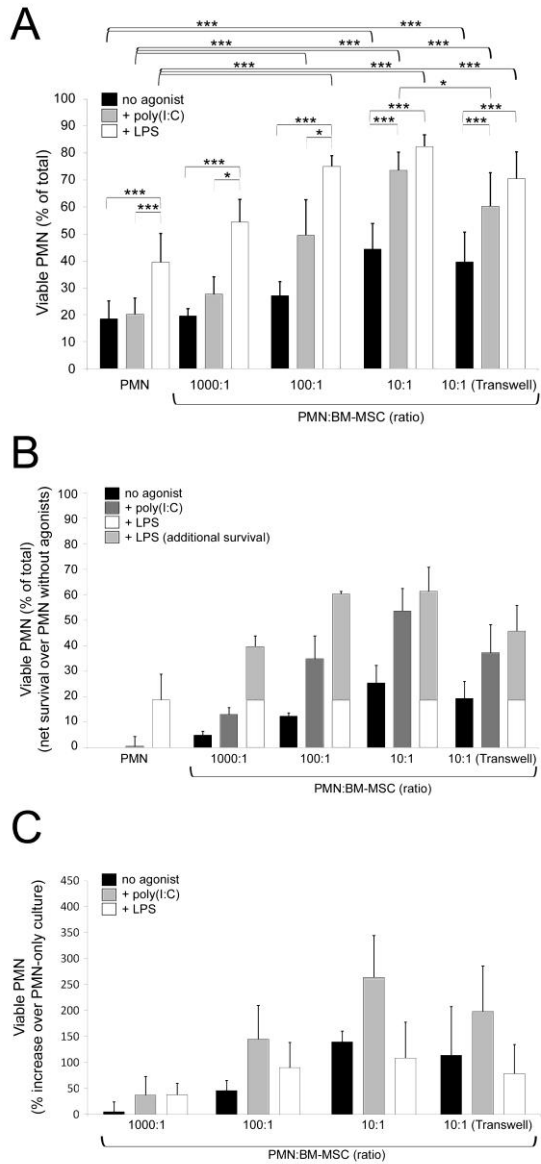
RESULTS

BM-MSC exert a powerful pro-survival effect on neutrophils upon activation by TLR3- or TLR4-agonists

Highly purified populations of peripheral neutrophils 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 neutrophils were investigated after 20 hours of incubation, unless differently specified. As shown in **Figures 12-13**, neutrophil 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 $44.4 \pm 9.5\%$ ($P < 0.001$) (**Figure 12**). 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 (**Figure 14**). Cocultures performed by using Transwell® inserts yielded similar results, with neutrophil survival ranging from $22.9 \pm 12.3\%$ (in the absence of BM-MSC, *data not shown*) to $39.6 \pm 10.9\%$ ($P < 0.01$) (**Figures 12-13**). These results substantially confirm and extend previous observations aimed at defining whether resting BM-MSC could influence neutrophil viability¹¹⁰.

To subsequently investigate whether activated BM-MSC could exert enhanced modulatory effects on neutrophil survival, we performed the cocultures in the presence of poly(I:C), the specific ligand of TLR3, which is expressed and functional in BM-MSC²¹⁻²³ but not in neutrophils^{3,112} (**Figures 12-13**). Under these conditions, the survival rate of neutrophils was strongly increased and already significant at the 100:1 PMN:BM-MSC ratio ($49.5 \pm 13.2\%$, $P < 0.001$), but was maximum at the 10:1 ratio ($73.6 \pm 6.8\%$, $P < 0.001$) (**Figures 12-13**).

FIGURE 12. BM-MSC exert a powerful prosurvival effect on neutrophils under coculture conditions in the presence of either poly(I:C) or LPS.

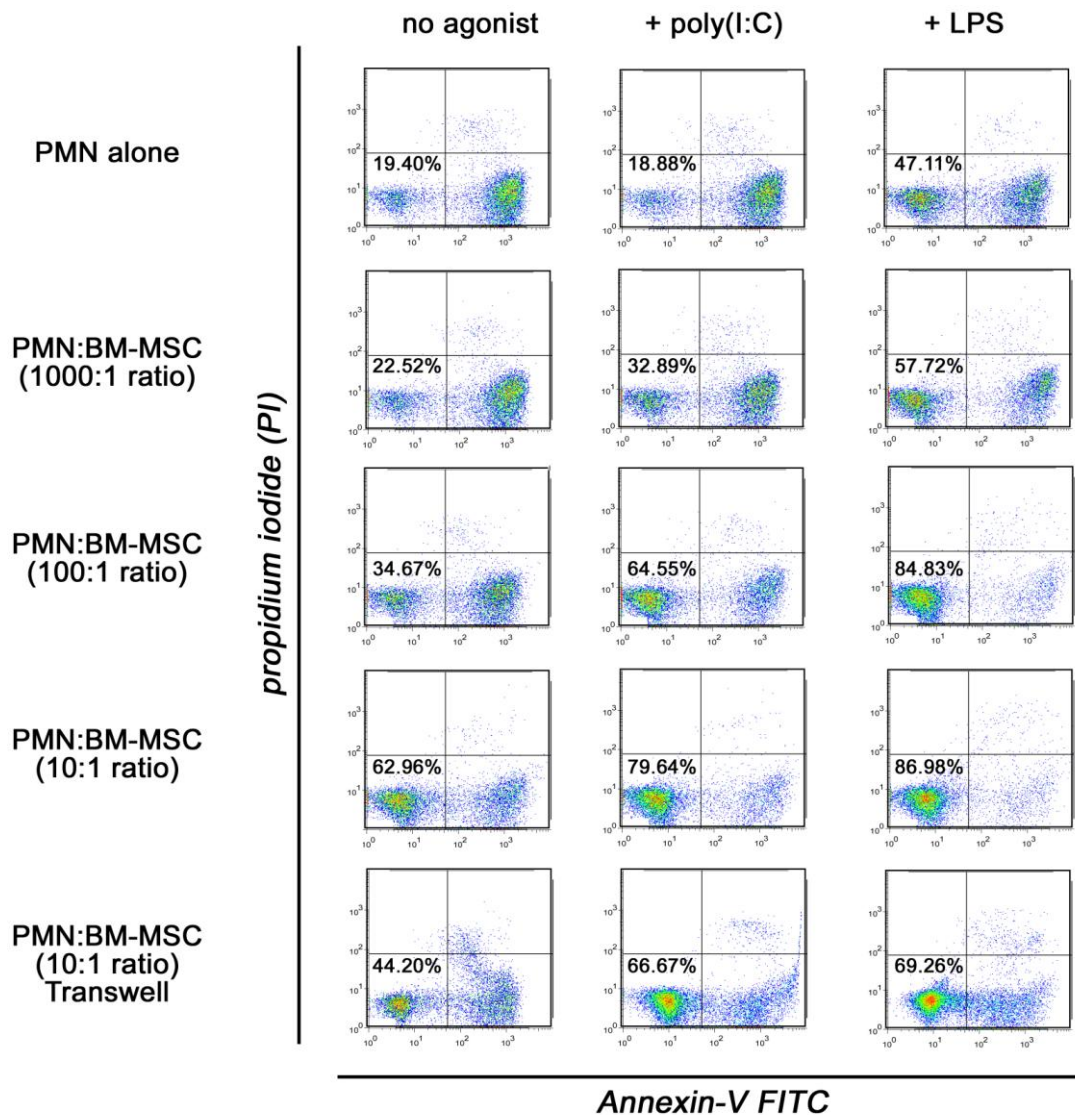


A. Bars indicate the survival levels of neutrophils (PMN) after coculture for 20 hours with or without BM-MSC either in direct contact or in Transwell®, at ratios ranging from 1,000:1 to 10:1, in the absence or presence of 100 µg/ml poly(I:C) or 100 ng/ml LPS. Neutrophils that were double negative by the Annexin-V/PI method (see *Materials & Methods*) were considered as viable: their percentage is reported as percentage of the total. Results are expressed as mean ± SD (n≥12 in all cases). *P* values <0.05 were considered as statistically significant (*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001).

B. Bars report the net prosurvival effect exerted by BM-MSC on neutrophils under the conditions reported in panel A. Data were calculated by considering the difference between the survival rate at 20 hours of neutrophils under the various coculture conditions over the survival rate of neutrophils cultured alone without TLR-agonists. The light grey fraction of the bars representing the cocultures treated with LPS better visualizes the net effect exerted by LPS-stimulated BM-MSC over the effect exerted by LPS by itself on neutrophils. Results are expressed as mean ± SD (n≥12 in all cases).

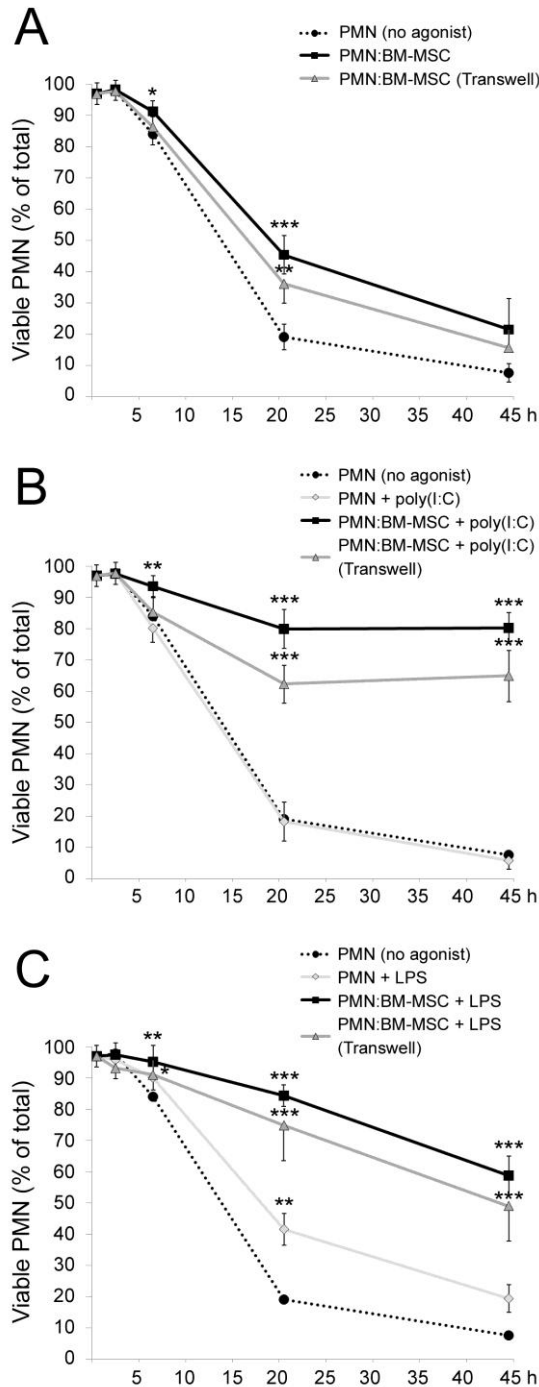
C. Bars report the percentage of increase of neutrophil survival in PMN:BM-MSC cocultures as compared to PMN-only cultures carried out with or without the corresponding TLR-agonists. Results are expressed as mean ± SD (n≥12 in all cases).

FIGURE 13. Representative Annexin-V/propidium-iodide (PI) plots displaying the neutrophil prosurvival effect exerted by poly(I:C)- or LPS-activated BM-MSC under coculture conditions.



The figure shows a representative experiment, performed as detailed in the legend of Figure 12. The percentage of double negative PMN, considered as viable, is shown in each condition.

FIGURE 14. Time course analysis of the viability of neutrophils during their coculture with resting and activated BM-MSC.



Neutrophils (PMN) were cocultured with or without BM-MSC as detailed in the legend of Figure 12 and harvested at different time points (0, +2, +6, +20 and +44 hours) to measure their viability. Double negative neutrophils with the Annexin-V/PI method were considered as viable and their percentage is reported as percentage of the total. Results are expressed as mean \pm SD (n=3).

Panel A displays the survival in cultures without TLR-agonists, while Panel B and Panel C display cultures with the addition of poly(I:C) and LPS, respectively.

Results was statistically compared to PMN cultures carried out without TLR-agonists, and *P* values <0.05 was considered as statistically significant (*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001).

Notably, at 10:1 ratio the degree of neutrophil survival was maintained at very high levels up to 44 hours (**Figure 14**), even under Transwell® conditions ($60.2 \pm 12.4\%$, $P < 0.001$) (**Figures 12-14**), 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^{21-23,111} and neutrophils^{3,112} express functional TLR4, the specific LPS receptor⁵⁹, and do respond to its ligands. In fact, LPS itself, in the absence of BM-MSC, significantly delayed neutrophil apoptosis (**Figures 12-14**), in line with the literature^{3,55,65}. Nonetheless, we further observed that neutrophil survival was additionally increased in LPS-treated PMN:BM-MSC cocultures, being apparently more pronounced than under poly(I:C)-treatment ($75.0 \pm 4.1\%$, $P < 0.001$ at the 100:1 ratio; $82.4 \pm 4.4\%$, $P < 0.001$, at the 10:1 ratio) (**Figures 12-13**). However, LPS activates neutrophils 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 eventually resulted more efficient than LPS-stimulated BM-MSC in protecting neutrophil viability, when considering the net protective effects over the basal viability of neutrophils cultured without TLR-agonists (**Figure 12B**), as well as the percentage of increased neutrophil survival observed in PMN:BM-MSC cocultures as compared to PMN-only cultures, with or without the corresponding TLR-agonists (**Figure 12C**).

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 (**Figure 14C**). On the other hand, Transwell® experiments proved that soluble factors were greatly responsible for the protection of neutrophil survival also in the case of LPS-treated PMN:BM-MSC cocultures (**Figure 12** and **Figure 13**).

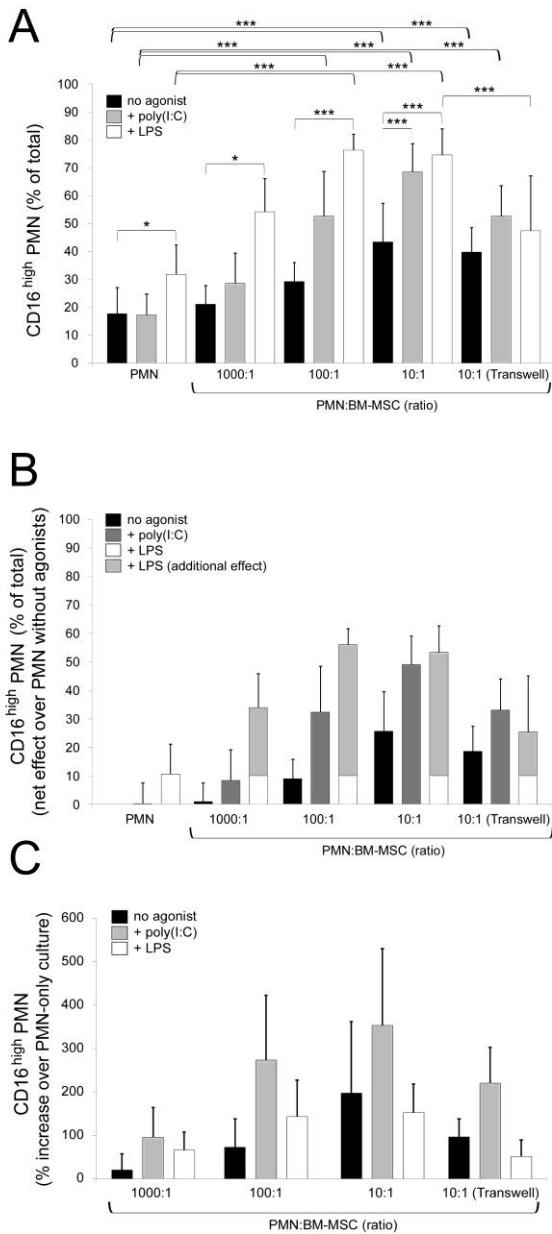
Taken together, these data show that BM-MSC activated with agonists for TLR3 and - less efficaciously - TLR4, delay neutrophil apoptosis much more significantly than resting BM-MSC.

Immunophenotypic changes by neutrophils after coculture with resting or TLR-activated BM-MSC

As previously described, CD16 (Fc γ R-III) can be reliably used as a surrogate marker of neutrophil viability¹¹⁵; accordingly, we observed that the percentage of neutrophils retaining high levels of CD16 expression (CD16^{high} PMN) matched the percentage of viable neutrophils under all coculture conditions (**Figures 15-16**). On the other hand, CD11b is typically modified by neutrophil activation^{107,114}, and was used in our study as a marker of the activation status of neutrophils.

As expected, neither the percentage of CD16^{high} neutrophils (**Figures 15-16**), nor the expression of CD11b (**Figures 17-18**) changed when neutrophils were cultured alone in the presence of poly(I:C). By contrast, the percentage of CD16^{high} neutrophils was significantly higher when neutrophils were cultured in the presence (43.37% \pm 13.92%) rather than in the absence (17.74% \pm 9.38%; P<0.001) of BM-MSC, at the 10:1 coculture ratio (**Figures 15-16**). A significant increase of CD16^{high} neutrophils was observed by adding either poly(I:C) (68.54% \pm 10.09%; P<0.001) or LPS (74.56% \pm 9.43%; P<0.001) to PMN:BM-MSC cocultures, similarly to what observed regarding the prosurvival effect. These data were comparable to the results obtained under Transwell® conditions (**Figures 15-16**).

FIGURE 15. Poly(I:C) or LPS-activated BM-MSC maintain higher levels of CD16 expression by neutrophils in coculture.



Neutrophils (PMN) were cultured as detailed in the legend of Figure 12 and then analyzed for CD16 expression by FACS analysis (see *Materials&Methods*).

A. Bars refer to the percentage of neutrophils maintaining a high level of CD16 expression (CD16^{high} PMN, see *Materials & Methods*) under the conditions detailed in Figure 12. Results are expressed as mean \pm SD ($n \geq 12$ in all cases).

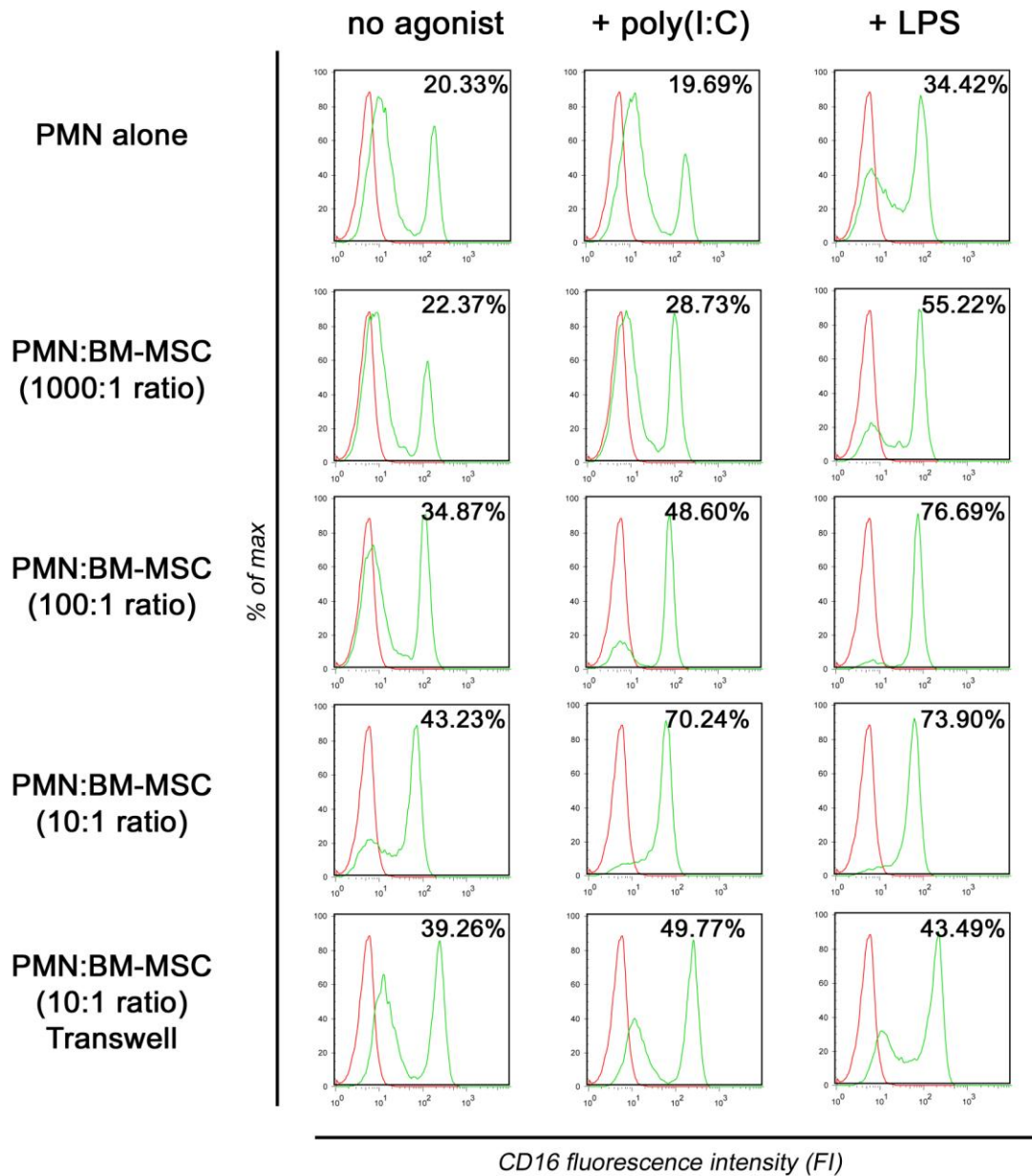
P values < 0.05 were considered as statistically significant (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

B. Bars report the net protective effect on neutrophil CD16 expression exerted by BM- MSC, under the conditions detailed in Figure 12, as calculated by considering the difference between the level of CD16 expression at 20 hours in the various coculture conditions over the level expressed by neutrophils cultured alone without TLR-agonists.

As in Figure 12B, the light grey fraction of the bars representing the cocultures treated with LPS better visualizes the net effect exerted by LPS-stimulated BM- MSC over the effect exerted by LPS by itself on neutrophils. Results are expressed as mean \pm SD ($n \geq 12$ in all cases).

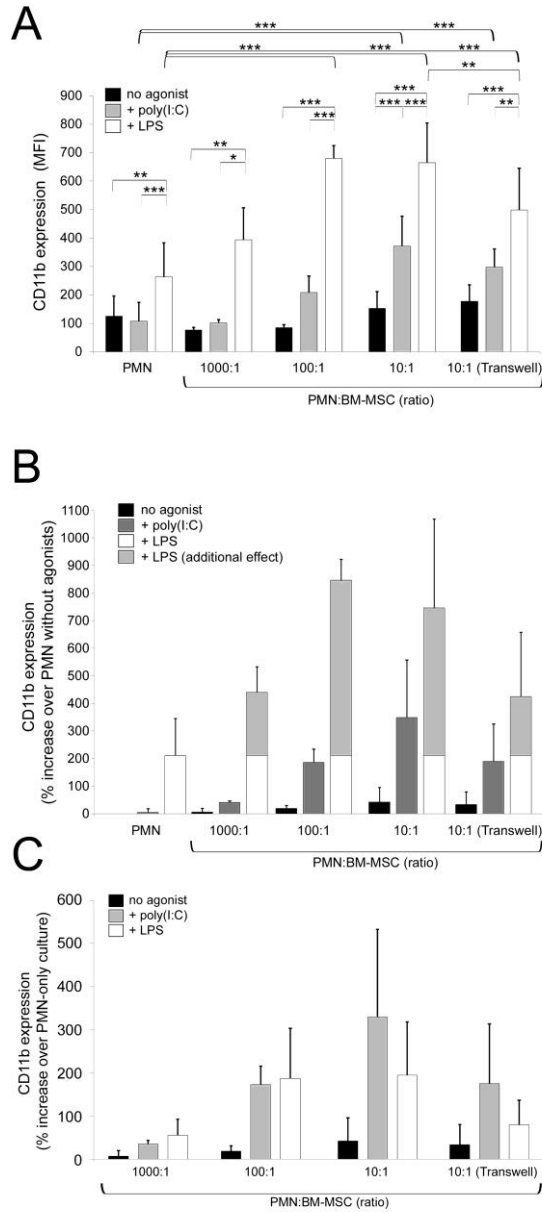
C. Bars report the percentage of increase of CD16^{high} PMN in PMN:BM- MSC cocultures as compared to PMN-only cultures realized with or without the corresponding TLR- agonists. Results are expressed as mean \pm SD ($n \geq 12$ in all cases).

FIGURE 16. Representative plots displaying the levels of CD16 expression in neutrophils after coculture with poly(I:C)- or LPS-activated BM-MSC.



The figure shows a representative experiment, performed as detailed in the legend of Figure 12. The percentage of PMN characterized by high level of CD16 expression (CD16^{high} PMN) is shown in each condition.

FIGURE 17. BM-MSC strongly upregulate neutrophil CD11b expression under coculture conditions in the presence of either poly(I:C) or LPS.



Neutrophils (PMN) were cultured as detailed in the legend of Figure 12 and then analyzed for CD11b expression by FACS analysis (see *Materials & Methods*).

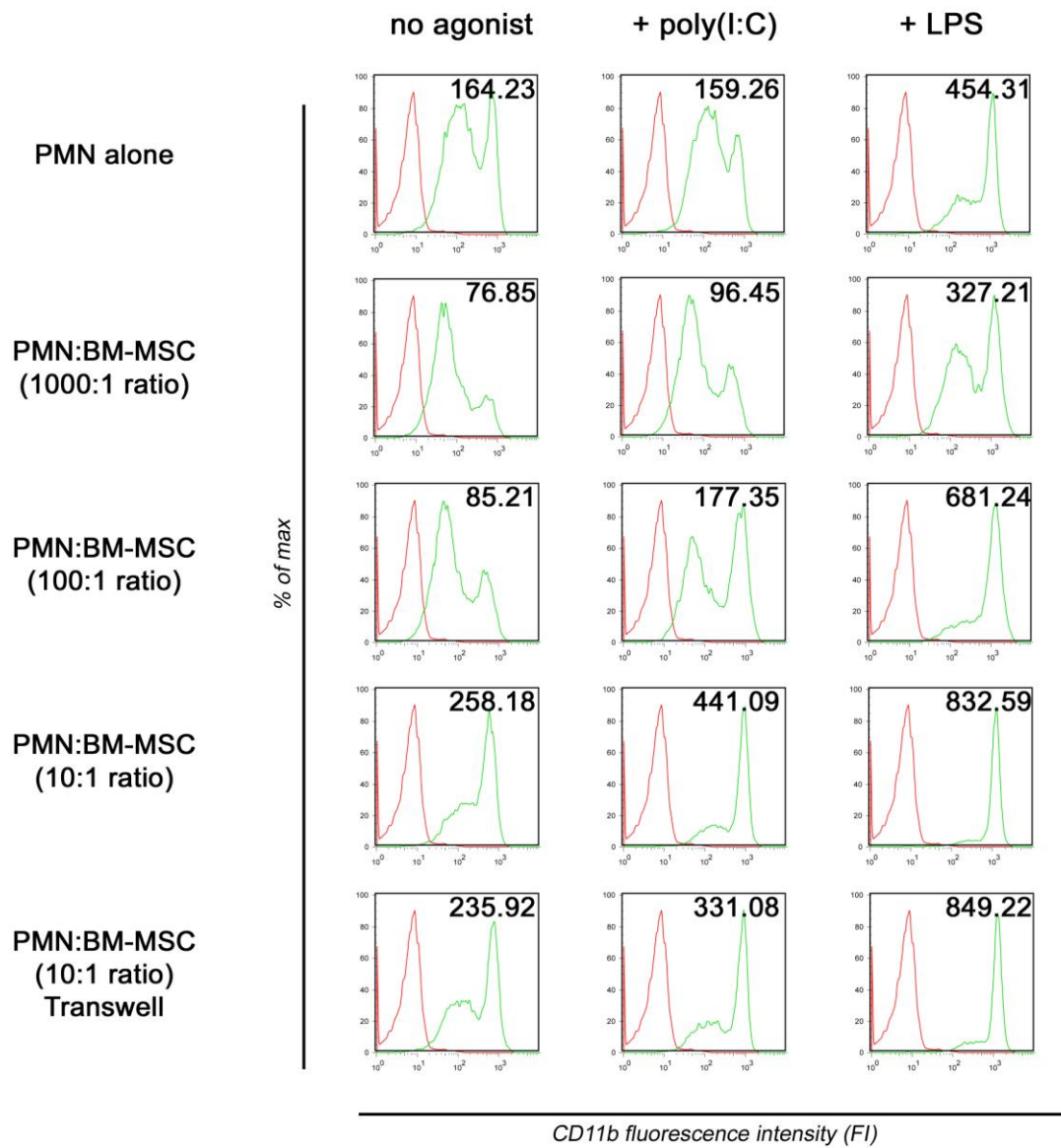
A. Bars indicate the geometric mean of fluorescence intensity (MFI) \pm standard deviation (SD) of CD11b in neutrophils (PMN) under the conditions detailed in Figure 12 ($n \geq 12$ in all cases). P values < 0.05 were considered as statistically significant (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

B. Bars report the geometric mean \pm SD of the percentage of increase on CD11b expression exerted by BM-MSC under the conditions detailed in Figure 12 ($n \geq 12$ in all cases). The increase was calculated by considering the difference at 20 hours between the levels of CD11b expression observed in neutrophils cultured in the various conditions and neutrophils cultured alone without TLR agonists, and it was expressed as percentage of the level of CD11b expression observed in neutrophils cultured alone without TLR agonists.

As in Figure 12B, the light grey fraction of the bars representing the coculture treated with LPS better visualizes the net upregulatory effect exerted on CD11b expression by LPS-stimulated BM-MSC over the effect exerted by LPS by itself on neutrophils.

C. Bars report the percentage of increase of CD11b expression levels in PMN:BM-MSC cocultures as compared to PMN-only cultures carried out with or without the corresponding TLR-agonists. Results are expressed as mean \pm SD ($n \geq 12$ in all cases).

FIGURE 18. Representative plots displaying the levels of CD11b expression in neutrophils after coculture with poly(I:C)- or LPS-activated BM-MSC.



The figure shows a representative experiment, performed as detailed in the legend of Figure 12. The geometric mean of CD11b fluorescence intensity (MFI) is shown in each condition.

On the other hand, the expression level of CD11b was unchanged by coculturing neutrophils with untreated BM-MSC, regardless of coculture ratios and the use of Transwell® inserts (**Figures 17-18**). By contrast, CD11b was significantly upregulated if poly(I:C) was added to PMN:BM-MSC cocultures (**Figures 17-18**). Similarly, the direct upregulatory effects of LPS on the percentage of CD16^{high} neutrophils and CD11b expression levels in neutrophils cultured without BM-MSC were also greatly amplified by BM-MSC (**Figures 15-16**), 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 panels **B** and **C** of **Figure 12**, aimed to detect the net effects of the cocultures as compared to the effect of LPS itself on neutrophils cultured alone (**Figures 15B-C** and **17B-C**). Interestingly, similar results concerning the percentage of CD16^{high} neutrophils and CD11b expression level were obtained under Transwell® conditions in LPS-activated PMN:BM-MSC cocultures (**Figure 15** and **Figure 17**). However, the higher variability observed in cocultures using Transwell® inserts partially limited the statistical significance of the immunophenotypic changes observed.

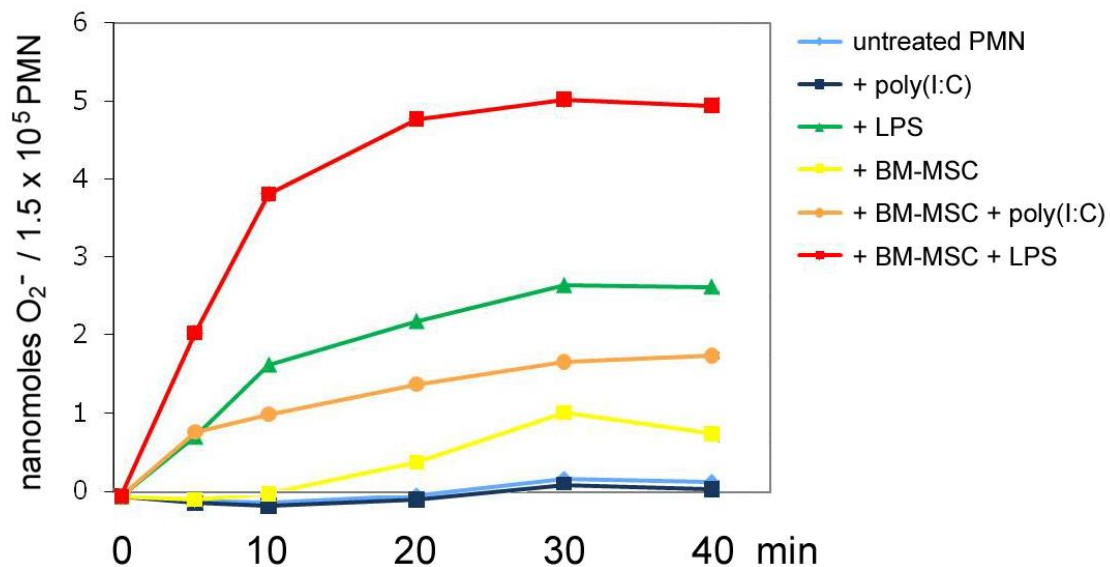
Taken together, these data demonstrate that TLR3- and TLR4-stimulated BM-MSC better preserve neutrophils expressing high-intensity CD16 and CD11b than resting BM-MSC.

Coculture with BM-MSC “primes” neutrophils for increased respiratory burst ability

The coculture with BM-MSC modified also the capacity of neutrophils of producing superoxide anion (O_2^-) in response to fMLF (**Figure 19**). Accordingly, while neutrophils cultured for 20 hours in the absence of BM-MSC and then stimulated with fMLF for up to 40 minutes resulted unable to release O_2^- (**Figure 19**), they properly

responded to fMLF challenge if preincubated for 20 hours with LPS, but not with poly(I:C) (**Figure 19**). Remarkably, neutrophils 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 poly(I:C) or (at higher levels) LPS (**Figure 19**).

FIGURE 19. Coculture with BM-MSC primes neutrophils for increased respiratory burst.



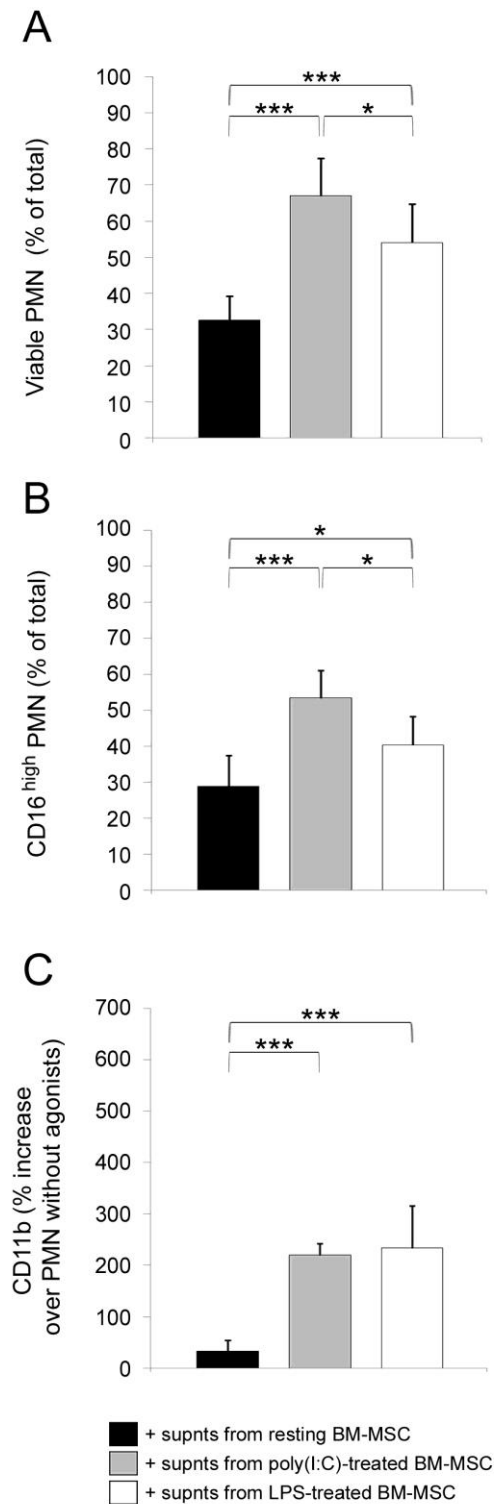
Neutrophils (PMN) were cocultured for 20 hours with or without BM-MSC at a 10:1 ratio, in the absence or presence of 100 $\mu\text{g/ml}$ poly(I:C) or 100 ng/ml LPS. Superoxide anion (O_2^-) production in response to 100 nM fMLF was then estimated by the cytochrome C reduction assay of triplicate samples. Absorbance at 550/468 nm was recorded every 5 minutes for the times shown. O_2^- production was calculated in nanomoles/ 1.5×10^5 PMN/minute using 24.5 mM as extinction coefficient. The figure shows a representative experiment out of three performed with similar results.

BM-MSC-conditioned supernatants virtually reproduce all the effects observed in PMN:BM-MSC cocultures

By culturing neutrophils 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 neutrophil survival and phenotypic changes (**Figure 20**). 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⁺ neutrophils (**Figure 20**). It is worth to note here that neutrophils were pretreated with a specific TLR4 blocking antibody¹¹⁶ prior to 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 **Figure 21**.

Overall, these data confirm that soluble factors contribute to mediate the modulatory effects exerted on neutrophils by resting or TLR-activated BM-MSC.

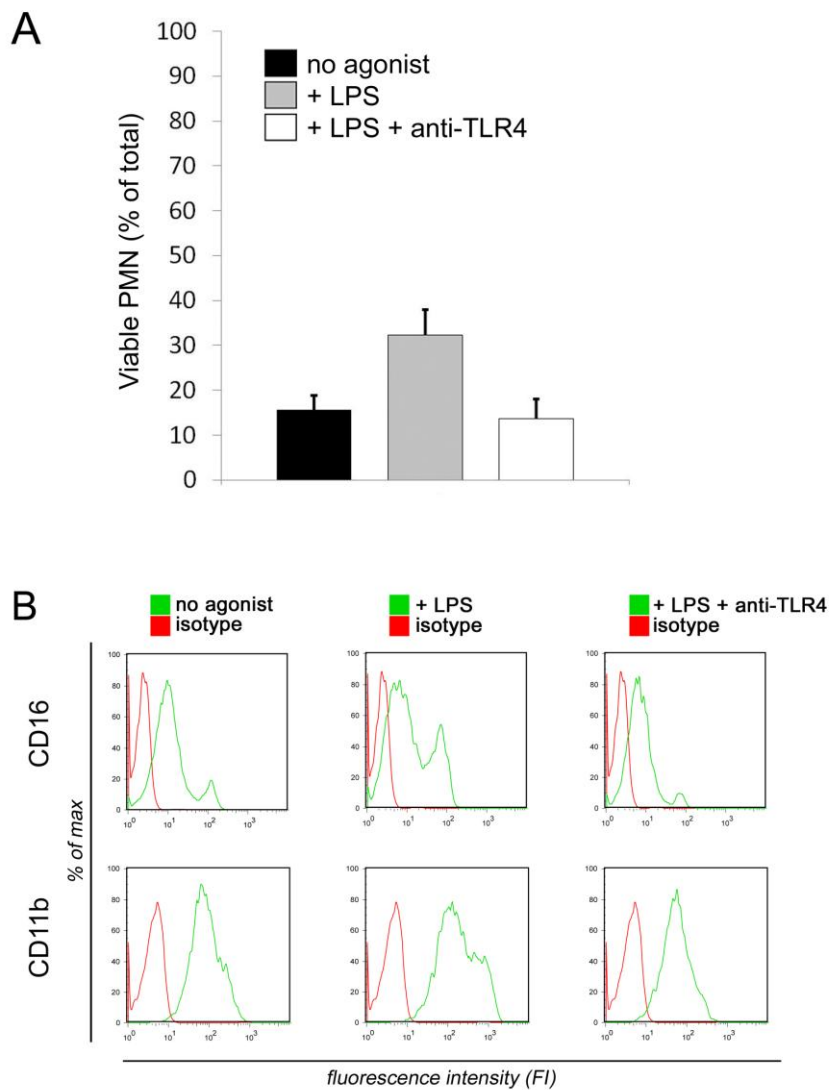
FIGURE 20. Effects of BM-MSC-conditioned supernatants on neutrophil viability and expression of CD16 or CD11b.



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 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 TLR-agonists. All results are expressed as mean ± SD (n=6).

FIGURE 21. Evaluation of the efficacy of the anti-TLR4 neutralizing mAbs to block LPS-mediated effects.



Neutrophils were cultured in regular medium (RPMI supplemented by 10% FBS and 1% penicillin/streptomycin) for 20 hours; apoptosis (A), CD16 (B) or CD11b (C) expression were then measured (see *Materials & Methods*). Results were compared to those obtained by adding 100 ng/ml LPS with or without 30-minute pre-incubation of neutrophils with 10 μ g/ml anti-TLR4 mAbs. A. Bars represent double negative neutrophils by the Annexin-V/PI method (see *Materials & Methods*), which were considered as viable: their percentage has been reported as percentage of the total. Results are expressed as mean \pm SD (n=3). B. CD16 and CD11b expression by neutrophils at the end of culture. Histogram of relative Fluorescence Intensity (FI) are shown (green lines) and compared to their isotype control (red lines).

IL-6, IFN- β and GM-CSF are primarily responsible for the effects on neutrophil survival and CD11b expression mediated by BM-MSC

We then measured a number of cytokines known to be involved in neutrophil 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, which both 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 phosphorylation¹¹⁷ (**Figure 22**). 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 NF- κ B and MAP kinase signalling pathways, as expected (**Figure 22**).

To identify which of the cytokines detected were responsible for neutrophil survival and activation under coculture conditions, we subsequently cultured neutrophils 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- γ and the related isotype matched controls. Both the protective effect on neutrophil 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 findings¹¹⁰. By contrast, the effects obtained with supernatants from poly(I:C)-treated BM-MSC were almost completely neutralized by the simultaneous use of anti-IL-6R, anti-GM-CSF and anti-

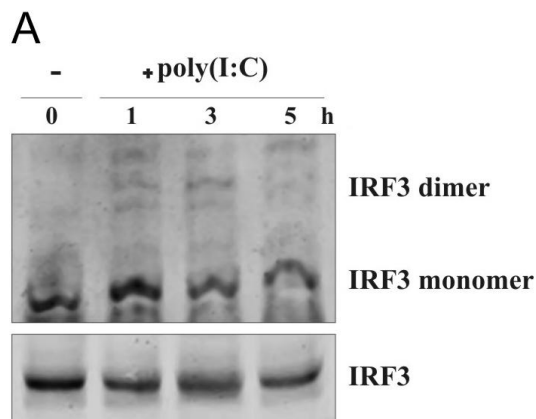
type I IFN-R mAbs, which, if used individually, produced only a limited, partial block (Figure 23). Interestingly, the effects of supernatants from LPS-treated BM-MSC were significantly reverted, although not completely, by anti-GM-CSF mAbs only, being all the other antibodies totally ineffective (Figure 23). Isotype controls did not exert any effect under all stimulatory conditions (*data not shown*).

TABLE 1. Cytokine released by BM-MSC activated by poly(I:C) or LPS.

<i>Cytokine</i>	<i>no agonist</i>	<i>+ poly(I:C)</i>	<i>+ LPS</i>
<i>IL-6 (ng/ml)</i>	1.3 ± 0.19	22.1 ± 0.62	14.2 ± 0.91
<i>IL-8/CXCL-8 (pg/ml)</i>	48.5 ± 21.2	2873.3 ± 6.3	2846.1 ± 4.4
<i>IFN-α (U/ml)</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
<i>IFN-β (U/ml)</i>	<i>n.d.</i>	5.964 ± 0.2	<i>n.d.</i>
<i>IFN-γ (U/ml)</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
<i>G-CSF (pg/ml)</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
<i>GM-CSF (pg/ml)</i>	<i>n.d.</i>	22.27 ± 19.5	21.35 ± 7.3
<i>TNF-α (pg/ml)</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>

IL-6, IL-8/CXCL-8, IFN-α, IFN-β, IFN-γ, G-CSF, GM-CSF and TNF-α were measured by specific ELISA in supernatants harvested from 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. *n.d.* not detectable.

FIGURE 22. Activation of the IRF3/STAT1 signaling pathway in poly(I:C)-treated BM-MSC.



BM-MSC were cultured with or without 100 $\mu\text{g/ml}$ poly(I:C) or 100 ng/ml LPS for the times indicated, prior to lysis for Western Blot analysis.

Panels A and B show that the stimulation of BM-MSC with poly(I:C) results in a transient IRF3 dimerization followed by a STAT1 tyrosine phosphorylation. Panel C shows that stimulation of BM-MSC with LPS results in the strong activation of both NF- κB and MAP kinase signaling pathways, as determined by both p38 and ERK phosphorylation, and I $\kappa\text{B}\alpha$ degradation, respectively.

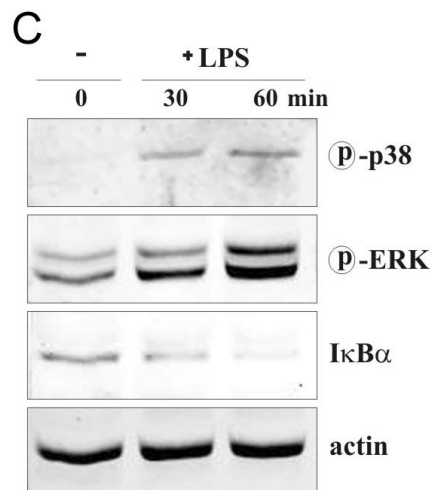
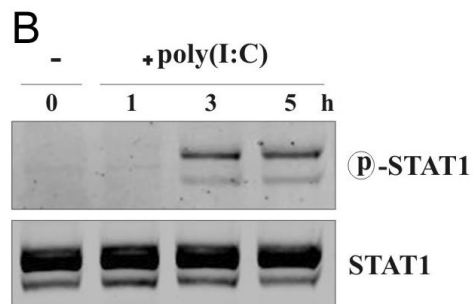
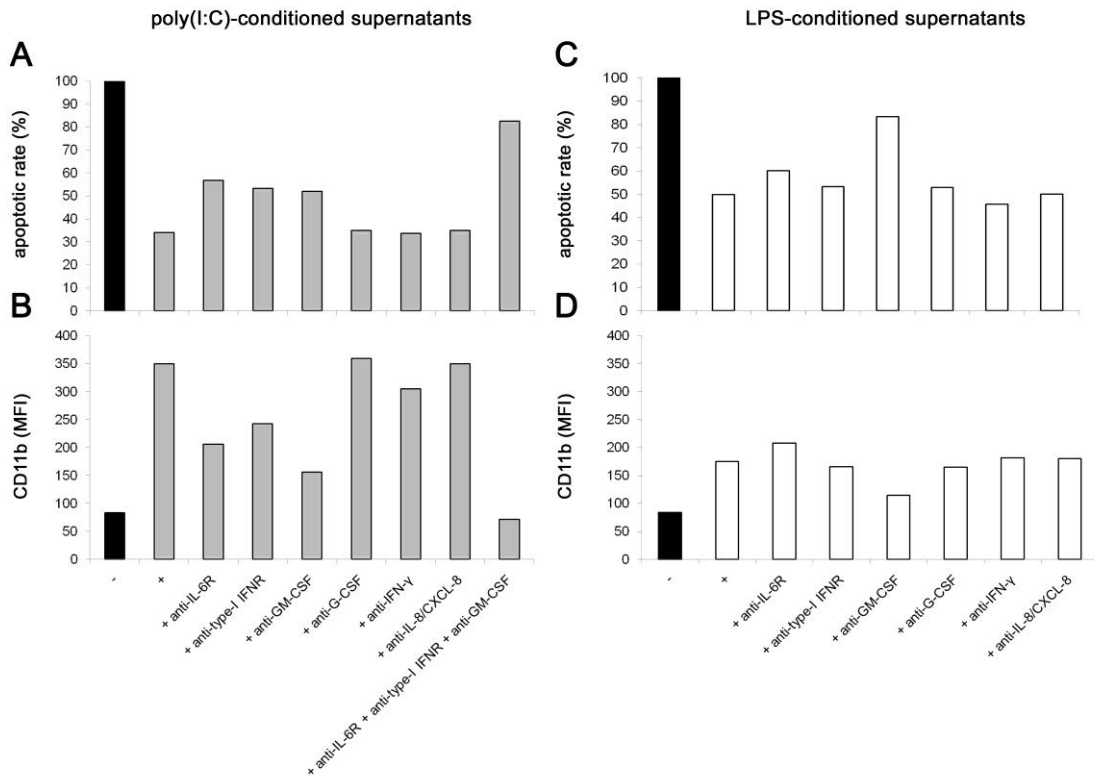


FIGURE 23. Poly(I:C)-activated BM-MSC modulate neutrophil apoptosis and CD11b expression through the release and combined action of IL-6, GM-CSF and IFN- β .

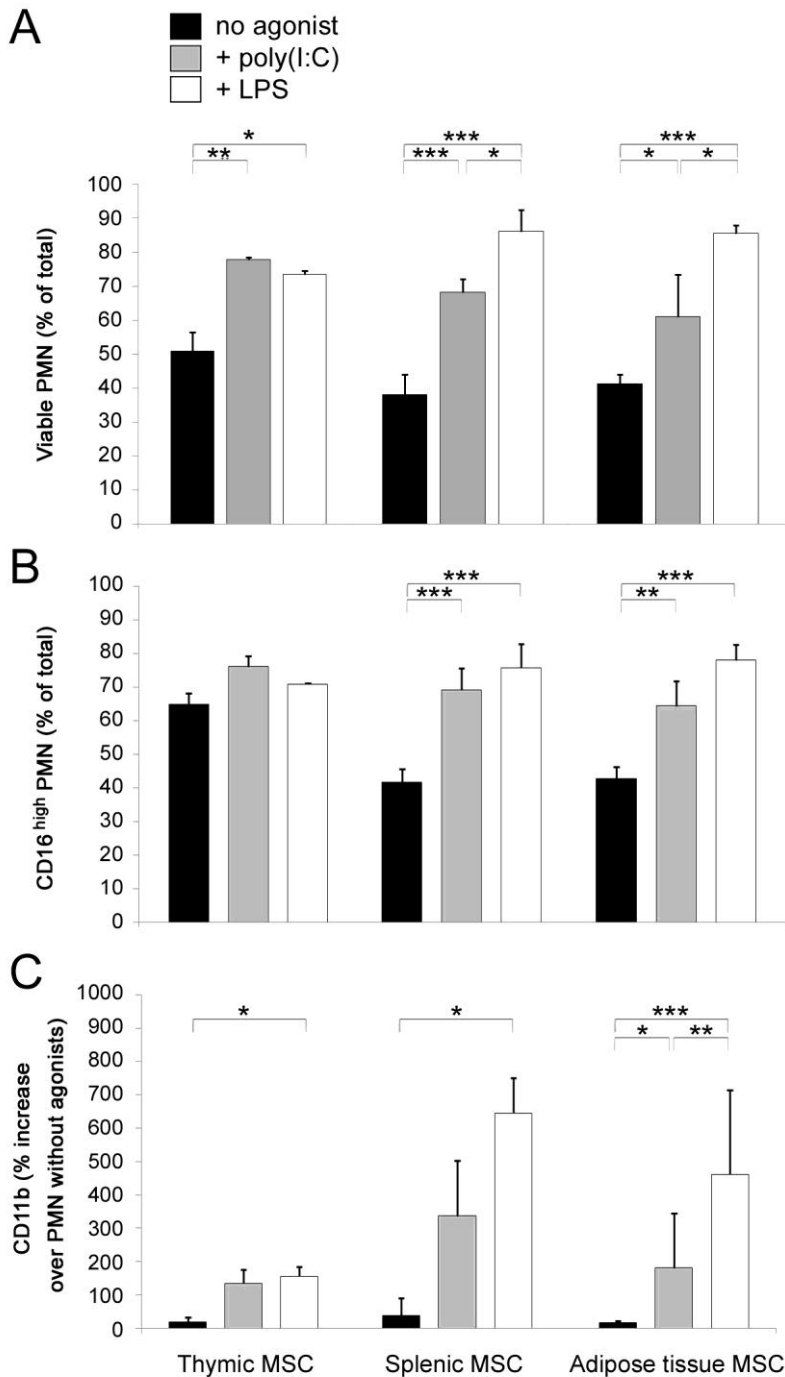


A-D. Human neutrophils (PMN) were cultured for 20 hours in supernatants collected from poly(I:C)- (A, B) or LPS-stimulated BM-MSC (C, D), in the presence or absence of 10 μ g/ml neutralizing mAbs (or appropriate isotype controls, *not shown*) directed towards IL-6R, type-I IFNR, GM-CSF, G-CSF, IFN- γ and IL-8/CXCL-8, either alone or in combination. In all conditions involving the use of supernatants from LPS-activated BM-MSC (C, D), neutrophils were preincubated for 30 minutes with anti-TLR4 mAbs (see *Materials & Methods*), prior to further culture. The degree of apoptosis (A, C) was compared to that observed in PMN culture performed in regular medium (see *Materials & Methods*) without TLR-agonists (apoptotic rate); the expression of CD11b (B, D) was measured as geometric mean fluorescence intensity (MFI). 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 (X): supernatant-treated cells with no neutralizing antibodies.

MSC from thymus, spleen and adipose tissue mimic the effects exerted by BM-MSC on neutrophils

MSC 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 MSC behaved like BM-MSC in terms of either their protective effect on neutrophil survival (**Figure 24**) or immunophenotypic changes (**Figure 24**), both at resting conditions and after TLR3- or TLR4-engagement, at least at the 10:1 PMN:MSC coculture ratio.

FIGURE 24. MSC from thymus, spleen and adipose tissue mimic the effects that BM-MSc exert on neutrophils.



A-C. Neutrophils (PMN) were cocultured for 20 hours at a 10:1 ratio with MSC isolated from thymus, spleen and adipose tissue under the same conditions used for BM-MSc and detailed in the legend of Figure 1. Survival (A), CD16^{high} fraction (B), and CD11b expression (C) were then analyzed by flow cytometry, as detailed in the *Materials & Methods* and in the legend of Figure 12. Results are expressed as mean \pm SD (n=3 in all cases). *P* values <0.05 were considered as statistically significant. (*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001).

DISCUSSION

In this study, we aimed at exploring under coculture conditions whether TLR-activated MSC could exert different modulatory effects on neutrophils 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 towards human neutrophils, which do not express TLR3 and do not respond to its ligands^{3,112}. As control, we analyzed LPS-stimulated neutrophils in coculture with BM-MSC, always carefully considering that both BM-MSC and neutrophils express the functional TLR4^{21-23,111}.

TLRs belong to the pattern recognition receptor (PRR) system that has multiple and pleiotropic functions, including the triggering of neutrophils during inflammation³. Also BM-MSC express several TLRs, capable of activating specific responses^{21-23,103,111}: for instance, while TLR2 maintains BM-MSC in their undifferentiated state, without affecting their immunomodulatory properties⁹⁷, both TLR3 and TLR4 influence their response to stress and migration^{22,111}, as well as regulate their immunomodulatory effects towards activated T lymphocytes^{21,100,103}. In addition, TLR3 and/or TLR4 engagement enhances BM-MSC production of IL-1 β , IL-6 and chemokines, such as IL-8/CXCL8, IP10/CXCL10, monocyte chemotactic protein (MCP)-1 and CCL5^{21-23,97-98,111}. Finally, TLR3-triggering seems to mediate, under specific conditions, the MSC polarization towards the inhibitory phenotype, while TLR4-activation would drive MSC towards the opposite pro-inflammatory status¹⁰³.

It was recently shown that preliminary results about the effects of TLR stimulation in neutrophils may have been influenced by the presence of residual

“contaminating” cells (mainly monocytes) in PMN preparation obtained after standard physical and chemical separation¹. To avoid this potential bias, several groups have added an immunomagnetical depletion step to highly purify neutrophil preparations prior to their use in experiments. The potential advantages of this approach has been pointed out by some recent publications^{7,53,107,118-119}. For instance, previous reports of increased survival by eosinophils in response to LPS were demonstrated to actually be an indirect effect of LPS on contaminating monocytes, rather than on eosinophils: the latter, in fact, do not express TLR4, and can not respond to LPS¹¹⁸. At the same time, contaminating cells, i.e. monocytes and eosinophils, may partially account also for the wide range of constitutive neutrophil apoptosis reported by unrelated studies. When analysing a series of factors independently acting on neutrophil apoptosis, Sabroe and colleagues¹¹⁹ found “*LPS addition*” and the “*presence of a residual percentage (<5% of mononuclear cells*” to be synergistically active in enhancing neutrophil survival in a CD14-depleted neutrophil preparation¹¹⁹.

Herein, using immunomagnetically-depleted, highly purified preparations of human neutrophils, we confirmed that in the absence of stimuli BM-MSC significantly prolong neutrophil survival in an IL-6-dependent manner, as previously described¹¹⁰. 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 neutrophil activation following coculture with resting BM-MSC, as previously described¹¹⁰. In particular, we did not observe any change either in the levels of neutrophil CD11b expression or in their respiratory burst capacity. Furthermore, no cytotoxic effect mediated by neutrophils towards the MSC monolayer during coculture was observed.

Strikingly, poly(I:C)-activated BM-MSC exerted a significantly greater protection of neutrophil 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 neutrophil respiratory burst ability and CD11b expression. Similar effects were detected in neutrophils 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 resulted partially related to the direct effect of LPS on neutrophil survival and immunophenotype. Thus, TLR3-stimulated BM-MSC appeared more efficient than TLR4-stimulated BM-MSC, as confirmed also by the comparative experiments of neutrophil 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 neutrophil survival and phenotypic pattern, thus strengthening the concept that TLR3 or TLR4 might regulate the interactions in different tissues between stromal cells and recruited neutrophils during inflammatory reactions.

Similar effects were obtained either under Transwell® conditions or by culturing neutrophils 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 effects⁵⁵, 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/CXCL-8 were found in supernatants from resting BM-MSC. These cytokines were even more concentrated in poly(I:C)- or LPS-derived supernatants, as previously reported²¹⁻²³.

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 neutrophil 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/CXCL-8 mAbs did not exert any change.

The latter data complement and extend the findings recently described by Brandau and colleagues¹¹¹, showing that supernatants harvested from parotid-derived MSC exposed to LPS for 4 hours contained many different inflammatory cytokines and were capable of delaying neutrophil 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 neutrophils, 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 the *Materials & Methods* section). Accordingly, the expression by neutrophils of CD64, a marker well-known to be upregulated after exposure to IFN- γ ¹²⁰, was never increased in neutrophils under any of the coculture conditions (*data not shown*). In addition, there is little data supporting the production of

IFN- γ by BM-MSc expanded from healthy donors, and the production of TNF- α by MSC is still a controversial issue^{22,121}. 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 neutrophil 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)¹¹⁷, a functional cross-talk between MSC and neutrophils could occur *in vivo* in the early response to viral infections. In addition, TLR3-activated MSC might influence neutrophil behaviour 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 strands²⁷⁻²⁸ that, in turn, may activate TLR3 in human dendritic cells²⁷, as well as in murine neutrophils and macrophages²⁸. 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 neutrophils *via* the production of TLR3-induced IL-8/CXCL-8 and CCL5^{21-23,111}.

Additionally, the demonstration that TLR3 ligation on MSC triggers a cascade of events that ultimately favour a prolonged neutrophil 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 arthritis¹²² might be explained by the shift of MSC towards an unexpected pro-inflammatory, neutrophil-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* TLR3¹²³: a similar phenomenon could occur following stimulation by autoantigens and/or endogenous ligands.

CONCLUSIONS

The data we report in this study add new information to 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/macrophages¹⁰³: in particular, that TLR4-primed MSC would exhibit a mostly pro-inflammatory profile with increased levels of molecules like IL-6, IL-8, or TGF β (and thus named as *MSC1*), whilst TLR3-primed MSC would develop the characteristics of immunosuppressive cells producing IL-10, IDO and PGE2 (and thus named as *MSC2*)¹⁰³.

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 pro-inflammatory by supporting the survival and function of neutrophils 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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