Thesis for the degree of Doctor of Philosophy (PhD)

MOLECULAR PATHOGENESIS OF RHEUMATOID ARTHRITIS: MYELOID SUPPRESSOR CELLS AT THE CROSSROADS OF NEW THERAPEUTIC AVENUES

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Table of Contents

1 ABBREVIATIONS

Ab:	antibody
ABCB1 (MDR):	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1
ACPA:	anti-citrullinated protein/peptide antibody
Ag:	antigen
ATIC:	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
BM:	bone marrow
CCL21:	chemokine (c-c motif) ligand 21
CCP:	cyclic citrullinated peptide
CCR6:	chemokine receptor 6
CD40:	cluster of differentiation 40
CEP:	citrullinated α-enolase peptide
CF:	citrullinated fibrinogen
CI:	confidence interval
CIA:	collagen-induced arthritis
cpm:	counts per minute
CTLA4*:*	cytotoxic T lymphocyte-associated protein 4
CV:	citrullinated vimentin
CYP1A2:	cytochrome P450
DHFR:	dihydrofolate reductase
DHODH:	dihydroorotate dehydrogenase
DMARD:	disease-modifying antirheumatic drug
DMEM:	Dulbecco's modified Eagle medium
ESR1:	estrogen receptor
FBS:	fetal bovine serum
FCGR:	Fcγ receptor
G-CSF:	granulocyte colony-stimulating factor
GM-CSF:	granulocyte/macrophage colony-stimulating factor
GWAS:	genome-wide association studies
HLA-DR:	human leukocyte antigen-DR
IBD:	inflammatory bowel disease
IgG:	immunoglobulin G
IL:	interleukin
IL23R:	interleukin 23 receptor
IL2RA:	interleukin 2 receptor alpha
IL2RB:	interleukin 2 receptor beta
IRF5:	interferon regulatory factor 5
ITPA:	inosine triphosphatase (nucleoside triphosphate pyrophosphatase)
LDA:	low disease activity
LTA:	lymphotoxin α
mAb:	monoclonal antibody
MAPK14:	mitogen-activated protein kinase 1
MBP:	myelin basic protein
MDSC:	myeloid-derived suppressor cell
MHC II:	major histocompatibility complex class II

MS:	multiple sclerosis
MTHFR:	methylene tretrahydrofolate reductase
MTX:	methotrexate
NO:	nitric oxide
NSAID:	non-steroidal anti-inflammatory drug
PADI4:	peptidylarginine-deiminase 4
PBMC:	peripheral blood mononuclear cell
PG:	proteoglycan (cartilage aggrecan)
PGIA:	PG-induced arthritis
PTPN22:	phosphatase non-receptor type 22
PTPRC:	receptor-type tyrosine-protein phosphatase C gene
QTL:	quantitative trait loci
RA:	rheumatoid arthritis
RF:	rheumatoid factor
RFC-1:	reduced folate career 1
RFC-1:	replication factor C (activator 1) 1
SCID:	severe combined immunodeficient/immunodeficiency
SE:	shared epitope
SEM:	standard error of mean
SF:	synovial fluid
SFC:	SF cell
SHMT:	hydroxymethyltransferase
SLC19A1:	solute carrier family 19 (folate transporter), member 1
SNP:	single nucleotide polymorphism
SSLP:	single sequence length polymorphism
STAT4:	Signal transducer and activator of transcription 4
TCR:	T cell receptor
Th:	T helper
TLDA:	TaqMan Low Density Array
TNFRSF1B:	tumor necrosis factor receptor superfamily, member 1b
TNF-α:	tumor necrosis factor α
TPMT:	thiopurine S-methyltransferase
TRAF1:	TNF receptor associated factor 1
TYMS:	thymidylate synthetase
WTCCC:	Wellcome Trust Case Control Consortium

2 INTRODUCTION

2.1 Brief description of the pathogenesis and therapy of rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune-inflammatory rheumatic disease accompanied by chronic, systemic synovitis. The prevalence of RA in the population is approximately 0.5–1%. Systemic inflammation eventually leads to joint destruction, disability and impaired quality of life [1]. The etiopathogenesis of RA is initiated by the "Bermuda triangle" of genetic and environmental/lifestyle-related risk factors, as well as autoimmunity [2-11].

In RA, the role of genetic factors have been approximated to 60% [6, 10, 12]. Class II major histocompatibility antigens/human leukocyte antigens (MHC, HLA-DR), along with non-HLA genes have been involved in disease pathogenesis [5, 6, 9-11, 13-15]. In the past few years, more consistent results of genetic associations have become available by current genome-wide association studies (GWAS) and other large-scale cohorts, such as the Wellcome Trust Case Control Consortium (WTCCC) database [10, 16, 17]. We have recently reviewed the genetic background of RA [10]. Without going into details, GWAS studies revealed two major genes in close association with susceptibility to RA. These alleles are *HLADR1* („shared epitope") and *PTPN22*. In addition, more than 30 single nucleotide polymorphism (SNPs) including *IL23R, FCGR, TRAF1, IRF5, CD40, STAT4, PADI4, CCR6, CCL21* have been associated with RA (Table 1) [10]. We have confirmed the association of *HLADRB1*, as well as *PTPN22* and *IL23R* SNPs in Hungarian RA patients [14, 18, 19].

Notably, HLA and some non-HLA genes have also been correlated with the production of autoantibodies to citrullinated proteins (ACPA), as well as tobacco smoking. Briefly, environmental and lifestyle-related factors (e.g. smoking, silica dust, anticontraceptives, caffeine, etc) may provoke protein citrullination, ACPA production and the onset of ACPA positive RA, as described in certain cohorts [4, 5, 9, 13, 20].

Some genes may be important for prognosis and outcome [4, 5, 13]. There may be a genetic predisposition of ACPA production [5, 9, 10, 13]. Some genes, primarily *HLADRB1* alleles, have been associated with ACPA positivity, as well as ACPA serum levels [20, 21]. In addition, different genes may determine ACPA seropositivity or seronegativity in RA patients (Table 2) [9, 13]. Moreover, ACPA positive RA may be more progressive and may exert worse prognosis than the seronegative disease [9, 13].

Table 1. The most important susceptibility alleles in RA pathogenesis [10]

Candidate gene	Encoded protein
HLA-DRB1	Class II human leukocyte antigen/major histocompatibility complex
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
TRAF1/C5	Tumor necrosis factor receptor-associated factor 1
STAT4	Signal transducer and activator of transcription 4
PADI4	Peptidylarginine-deiminase 4
IFR5	Interferon-related factor 5
FCGR	Fc gamma receptor
IL2RA, IL2RB	Interleukin 2 A and B
CD40	CD40
CCL21	CC chemokine ligand 21
CCR6	CC chemokine receptor 6

* See text for abbreviations and further explanation

Table 2. Genes that may determine ACPA seropositivity and seronegativity in RA

ACPA positive	ACPA negative
HLA-DRB1*01	HLA-DRB1*03
HLA-DRB1*04	HLA-DRB1*13
HLA-DRB1*15	IRF5
CTLA4	STAT4
STAT4	

* See text for abbreviations and further explanation

Genetic and environmental factors, as well as autoantibody (ACPA)-mediated autoimmunity trigger synovitis, the central inflammatory event in RA, as well as joint destruction [22, 23]. Several inflammatory cells and mediators are involved in this process. T cells play a crucial role in the initiation and perpetuation of synovitis [24, 25]. The genetic linkage between disease susceptibility and certain HLA-DRB1 molecules expressed by antigen-presenting cells corroborates the role of autoreactive T cells in RA pathogenesis [10]. In addition, T cell recognition of citrullinated autoantigens (autoAgs) is also unevitable for the initiation of RA-associated autoimmunity [25, 26]. Furthermore, the presence of native and citrullinated self proteins and isotype-switched antibodies (Abs) against self immunoglobulin G (IgG, i.e., rheumatoid factor, RF) in the majority of RA patients [5] may be the result of help to Ab-producing B lymphocytes provided by T helper (T_H) cells [24]. T lymphocytes

present in the arthritic joint mostly belong to the T_H1 and T_H17 subsets. These cells are considered to participate greatly to local tissue inflammation [24, 25, 27]. Neutrophils, that belong to the innate immune system, are major constituents in the RA synovial fluid (SF) [22, 28]. Although SF monocytes and granulocytes are able to substantially harm the joint structures via secretion of proteolytic enzymes, pro-inflammatory cytokines, and other inflammatory mediators [28], they may also impair joint T cells thus restricting their local expansion [23, 25].

Early diagnosis [29] and prompt, effective medication are needed to prevent joint damage, functional disability and unfavorable disease outcome [30, 31]. Optimally, complex pharmacological and non-pharmacological treatment is necessary within 3–6 months after disease onset. A very narrow "window of opportunity" is available in order to obtain remission or low disease activity, the major targets for RA therapy [30-32]. In addition to corticosteroids and non-steroidal anti-inflammatory drugs (NSAID), as symptomatic treatment, several traditional disease-modifying drugs (DMARD) including methotrexate (MTX), leflunomide, sulfasalazine, chloroquine and others, as well as numerous biologic and synthetic targeted therapies have become available during past years. For example, biologics are not only able to suppress inflammation and pain, but they can also slow down the disease process, prevent joint damage and comorbidities, such as cardiovascular disease, and improve musculoskeletal function and quality of life [30, 31, 33, 34]. Interestingly, certain gene profiles or signatures have also been associated with therapeutic drug responses (pharmacogenetics / pharmacogenomics) [35-39]. For example, SNPs in several genes involved in folate metabolism or cellular drug transport, such as *MTHFR, ABCB1 (MDR), ATIC, TYMS,* have been associated with efficacy or toxicity of MTX (Table 3) [10, 37-39]. In addition, polymorphisms in the *TNF, MAPK14, FCGR3A* and other genes may predict the efficacy of biologics, primarily TNF-α inhibitors (Table 4) [35, 37-40].

As mentioned earlier, not all patients respond well to therapy, therefore there is still an unmet need in RA management that facilitate further research and development of anti-arthritic compounds [30, 31]. While transplantation of autologous bone marrow (BM) or hematopoietic stem cells have been of great promise in both refractory RA [41] and therapy-resistant juvenile idiopathic arthritis [42], clinical remission in these patients is still insufficient. Further research is needed in order to develop new therapeutic modalities for RA and other autoimmune-inflammatory rheumatic diseases.

Table 3. Pharmacogenomics of traditional disease-modifying agents [10]

Drug	Gene	Variant	Clinical effect
Methotrexate	*SLC19A1 (RFC-1)*	G80A	Increased or unaffected efficacy
	MTHFR	C677T	Increased toxicity in most studied
	MTHFR	A1298C	Controversy regarding toxicity and efficacy
	SHMT1	C1420T	Increased toxicity
	ABCB1 (MDR1)	C3435T	Decreased efficacy
	TYMS	5'-UTR repeat element	Decreased efficacy, probably increased toxicity
	TYMS	3'-UTR deletion	Increased efficacy
	ATIC	C347G	Increased efficacy and toxicity in most studies
	IL1RN	IL-1RN*3	Decreased efficacy
Sulfasalazine	*NAT2*	NAT2*4	Increased toxicity in slow acetylators in most studied increased efficacy in rapid acetylators (?)
Leflunomide	*DHODH*	C19A	Increased efficacy and toxicity
	ESR1	SNP	Increased efficacy
	CYP1A2	CYP1A2*1 F	Increased toxicity
Hydroxychloroquine	*IL10*	A1082G	Increased efficacy
		C819T	
		C592A	
	TNF	A308G	Increased efficacy
Azathioprine	*TPMT*	TPMT*2, *3A, *3C	Increased toxicity
	ITPA	C94A	Increased toxicity

* See text for abbreviations and further explanation

Table 4. Pharmacogenomics of biologics [10]

Drug	Gene	Variant	Clinical effect
Anti-TNF agents	*TNF*	G308A	Increased efficacy in most studies
	TNF	A238G	Increased efficacy
	TNFRSF1B	T196G	Decreased or no effect on efficacy
	FCGR3A	Val158Phe	No effect on efficacy
	PTPRC	SNP	Increased efficacy
	MAPK14	SNP	Increased efficacy of anti-TNF antibodies (infliximab, adalimumab
Rituximab	*FCGR3A*	Val158Phe	Increased efficacy or no effect

* See text for abbreviations and further explanation. Large gene signature studies are not included.

2.2 The involvement of myeloid-derived suppressor cells in inflammation and arthritis

Recently, a heterogeneous population of immature myeloid cells with immunosuppressive capability, named myeloid-derived suppressor cells (MDSCs) have been described. Most research data originated from cancer studies [43-45], however, an increasing body of evidence supports the role of these cells in chronic inflammatory conditions. As the nomenclature suggests, MDSCs belong to the innate immune system and exert the capacity to suppress adaptive immune responses. Although the number of MDSCs is rather low in healthy individuals, the accumulation of these cells has been described in malignancies, infections, trauma, graft-versus-host reaction and autoimmunity. MDSCs do not represent a well-defined cell type, rather, they represent a collection of maturation-resistant "monocyte-" and "granulocyte-like" cells that develop from myeloid hemopoietic precursors. They are also heterogeneous in morphology and phenotype, and are capable of actively suppressing T cell responses. Recent studies have characterized the different subsets of MDSCs, their modes of action, and their possible role in a variety of autoimmune conditions, such as inflammatory bowel disease, alopecia areata, autoimmune uveoretinitis, multiple sclerosis, type I diabetes, as well as RA. The presence of MDSCs in cancer is detrimental, since they promote tumor cells to escape from immune mediated elimination. In contrast, in autoimmune diseases, such as RA, the suppressive capacity of MDSCs towards autoreactive T cells may open therapeutic options [43, 45].

In more detail, MDSCs were originally described in tumour-bearing mice and in humans with cancer more than two decades ago [43-45]. Ever since, the vast majority of data have been generated in cancer. However, accumulating evidence supports the role of these cells in chronic inflammatory states, such as bacterial or parasitic infections, traumatic stress, sepsis, transplantation, as well as autoimmune disorders, such as RA [46-50]. In RA and animal models of this disease, MDSCs interfere with synovial inflammation, T_H17 responses, as well as bone erosion [50, 51]. The mutual feature of the above mentioned disorders is the release of a broad array of soluble mediators (growth factors, inflammatory cytokines) affecting not only the target tissues and organs but also interfering with normal myelopoiesis in bone marrow (BM). Certain factors promote the expansion of MDSCs via stimulation of myelopoiesis. Some of these mediators inhibit myeloid cell differentiation and contribute to the congregation of MDSCs at sites of inflammation. As the microenvironment under different pathological conditions varies, the phenotypic and functional features of MDSCs

could also be rather diverse in different conditions [46, 47]. MDSCs are not considered as a homogenous cell population, rather a heterogeneous combination of monocyte, neutrophil and myeloid dendritic cell precursors. Yet, MDSCs form a single integrated system as their common feature is the capacity to suppress various immune responses [52]. Therefore, we may regard the MDSC network as a universal tool developed by nature to control immune responses under various pathological conditions [46, 52].

The subsets and the surface markers of MDSCs have been more precisely described in mice as all murine MDSCs express the common myeloid markers CD11b (the Mac-1 integrin) and Gr-1. Based on the use of anti-Gr-1 specific antibodies (Ly6C and Ly6G), binding to one of the two epitopes of Gr-1 molecule, the following subtypes have been identified: granulocyte-like $Gr1^{+}CD11b^{+}Ly6G^{+}Ly6C^{low}$ and monocytic $Gr1^{+}CD11b^{+}Ly6G^{-}Ly6C^{hi}$ MDSCs. These two major subsets may have different functions under pathological conditions. Although MDSCs in malignancies are responsible for inhibiting immunosurveillance, under autoimmune conditions, their immunsuppressive ability could be therapeutically utilized in order to limit further tissue damage [53, 54].

There are several protocols to generate MDSCs *in vitro* from murine BM precursors. Lutz et al [55] reported that immature DCs, cultured from BM with low dose of granulocyte/macrophage colony stimulating factor (GM-CSF) alone or with IL-4, can induce T cell unresponsiveness and prolonged cardiac allograft survival. In an animal model of autoimmune diabetes, Steptoe et al [56] could prevent disease development by transferring $Gr\text{-}1^{+}CD11b^{+}CD11c^{-}$ myeloid progenitor cells and $CD11c^{+}CD11b^{+}CD86^{lo}$ immature DCs. These cells were generated from BM precursors in the presence of GM-CSF and transforming growth factor-beta1 (TGF-β1). This cytokine mixture expands myeloid cells, but inhibits terminal differentiation of DCs. The presence of GM-CSF seems to be crucial for MDSC generation, however, the dose and treatment duration may influence success. In the study of Rössner et al [57], high dose of GM-CSF induced MDSC activity after 3-4 days, while low dose of GM-CSF required 8-10 days to generate immature DCs *in vitro*. These cells were able to suppress $CD4^{+}$ and $CD8^{+}$ T cell proliferation in the presence of interferon-gamma (IFN-γ) via intercellular contact and nitric oxide (NO) production. MDSCs could be generated not only from BM hematopoietic stem (HS) cells but from murine embryonic stem (ES) cells. According to the protocol of Zhou et al [58], two main subsets of MDSCs could be obtained: $Gr\text{-}1^{+}CD115^{+}F4/80^{+}$ monocytic, and a previously unreported granulocyte/macrophage progenitor-like $CD115^{+}Ly6C^{-}$ subtype. Both ES- and HS-derived MDSCs showed robust suppression of T cells via the production of NO and IL-10, as well as induction of regulatory

T (T_{REG}) cells. Furthermore, ES-MDSCs in an *in vivo* adoptive cell transfer system prevented graft-versus-host disease (GVHD). Highfill et al [59] described a promising method in order to generate a more potent MDSC population to prevent GVHD. These IL-4Rα$^+$ and F4/80$^+$ CD11b$^+$Ly6G^{lo}Ly6C$^+$ MDSC populations are generated from BM precursors using GM-CSF and IL-13. The suppressive action of these MDSC populations involved L-arginase-1 up-regulation. Another approach to generate MDSCs *in vitro* is to mimic the tumor microenvironment. Valenti et al [60] cultured CD14$^+$ monocytes, isolated from healthy donors, with microvesicules transporting cytokines and other biologically active molecules from advanced melanoma patients. Through this protocol, CD14$^+$HLA-DR$^{-/low}$ cells exerting TGF-β-mediated suppressive activity were obtained. In order to identify the most potent cytokine combination, Lechner et al [53, 54] cultured PBMCs, isolated from healthy donors, in the presence of different tumor cell lines. CD33$^+$ cells with potent suppressive ability could be best grown *in vitro* by administering GM-CSF and IL-6 and, secondarily, GM-CSF+IL-1β, prostaglandin-E2 (PGE_2), tumor necrosis factor-alfa (TNF-α) or vascular-endothelial growth factor (VEGF). While the possible soluble factors implicated in the generation of MDSCs are better and better characterized, there is still limited information available regarding their molecular signaling pathways. Marigo et al [61] reported that a potent IL-4Rα$^+$ MDSC population could be generated by a short administration of GM-CSF+G-CSF or GM-CSF+IL-6 cytokine combination. These cytokines involved a common molecular pathway entirely dependent on the C/EBPβ transcription factor. Thus, there are a number of protocols available to generate MDSCs *in vitro* in tumor models, transplantation, or in certain autoimmune disorders; however, none of these methods have been applied to RA or to animal models of arthritis so far. Therefore, in our studies, we explored whether *in vitro* generated MDSCs could be of therapeutic value in a mouse model of RA.

Proteoglycan-induced arthritis (PGIA) is an appropriate rodent model of RA induced by intraperitoneal injections of human cartilage proteoglycan (PG) in BALB/c mice. This model exerts most characteristics of human RA including genetics, immune responses, autoantibody production, as well as the production of inflammatory cytokines [62-64].

Our group previously identified a cell population with myeloid phenotype and biological capacity resembling MDSCs in the synovial fluid (SF) of mice with PGIA. The immunosuppressive ability of these cells was confirmed. Experiments using various inhibitors of MDSC-effector molecules revealed that the mechanism of suppression activity is via production of reactive oxygen species (ROS) and NO. The maturation of DCs was also suppressed by SF MDSCs through down-regulation of MHC-II and CD86 expression

resulting in the inhibition of (auto)-antigen presentation. During the phenotypical characterization procedure, we found that SF was dominated by $Gr1^{+}CD11b^{+}L6yG^{hi}Ly6C^{int/lo}$ (granulocyte-like) MDSCs, however, $Gr1^{+}CD11b^{+}L6yC^{hi}Ly6G^{neg/low}$ monocytic cells were also detectable. It is possible that the inflammatory microenvironment and the specific cytokine milieu (for e.g. GM-CSF, IL-6) present in the affected joints supports not only the recruitment of immature granulocytic precursors but may also conserve them in immature state with strong immunomodulatory capacity. These results imply that MDSCs in the SF could be exploited for therapeutic purposes [65].

Our previous study, as well as investigations by others suggest that RA SF may contain MDSCs that are capable to restrain the expansion of joint-infiltrating, therefore most likely pathogenic T cells. Our present studies represent the first step to study the role of MDSCs in controlling autoimmune T cell responses in RA.

3 AIMS

In this Ph.D. thesis, various aspects of the role of MDSCs are discussed, in context with the pathogenesis of autoimmune arthritis, as well as future prospects for therapy. For this purpose, we have performed *in vitro* and *in vivo* animal experiments, and *ex vivo* studies using human samples.

Our specific aims were as follows:

1. To develop an *in vitro* method for generating MDSCs similar to those found in SF.

2. To investigate the therapeutic effect of MDSCs in PGIA.

3. To identify and characterize MDSCs in the SF of patients with RA.

4 MATERIALS AND METHODS

4.1 Animal studies

4.1.1 Mice, immunization, and assessment of arthritis

Adult female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). Enhanced green fluorescent protein-lysozyme M transgenic (EGFP-LysM-Tg) mice [66] were back-crossed to the BALB/c background for 10 generations [65, 67]. Spleens of naïve PG-specific T cell receptor transgenic (PG-TCR-Tg) BALB/c mice recognizing a dominant epitope within the G1 domain of human PG [68] were used as a source of PG/G1-specific T cells. BALB/c mice with the severe combined immunodeficiency (scid) mutation (SCID mice) [67, 69] were purchased from the National Cancer Institute.

To induce arthritis, adult female wild type (wt) BALB/c mice were injected intraperitoneally (i.p.) with 100 μg of human PG protein [62] emulsified in dimethyl-dioctadecyl-ammonium bromide (Sigma-Aldrich, St Louis, MO, USA) adjuvant in sterile phosphate buffered saline (PBS) 3 times 3 weeks apart [70, 71]. PG was extracted from human cartilage as described previously [62, 65]. Cartilage was donated by patients undergoing joint replacement surgery. Written informed consent was obtained from each patient. Collection of surgical specimens was approved by the Institutional Review Board of Rush University Medical Center (Chicago, IL, USA). After the second injection of human PG, the limbs of immunized mice were examined for signs of arthritis. A standard visual scoring system (based on swelling and redness, ranging from 0 to 4 for each paw, 0-16 per mouse) was used for the assessment of disease severity. All experiments involving animals were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal studies were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center, Chicago, IL, USA (Permit Number: 11-046).

In this process the author participated in all cases along with Tibor T. Glant, who also participated in the assesment of the arthritis in the cell transfer experiments as an independent investigator.

4.1.2 Collection of serum and cells/organs from mice, and histology

Blood for cell analysis and measurement of anti-PG Ab titers was drawn from mice under deep anesthesia induced by intramuscular injection of a Ketamine-Xylazine cocktail. Mice were then euthanized by carbon dioxide inhalation, and spleen, BM, joint-draining lymph nodes (LNs) were collected. SF was harvested in heparin containing tubes from arthritic ankles, knees, and forepaws at the peak of the disease (inflammation score: 8-16 per mouse) after puncturing and gently pressing of the joints. Red blood cells were eliminated by hypotonic lysis and single cell suspensions were prepared from the harvested tissues and fluids.

For histology, hind limbs were dissected, fixed with formalin, decalcified, and embedded in paraffin. Sagittal sections, cut from the paraffin-embedded tissue, were stained with hematoxylin and eosin and examined under a Nikon Microphot light microscope (Nikon, Melville, NY, USA). Microphotographs of the ankle (tibio-talar) joints were prepared using a digital color CCD camera (Coolsnap; RS Photometrics, Tucson, AR, USA) and MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

The author performed all the experiments but Katalin Mikecz occasionally helped with the collection of specimes. The histology was performed with the help of Tibor T. Glant.

4.1.3 Generation of MDSCs and DCs from BM

MDSCs were generated from BM of naïve wt or EGFP-LysM-Tg BALB/c mice. Femurs and tibias were collected under aseptic condition, and BM was flushed out with sterile PBS. After red blood cell lysis, BM cells were counted (the number of cells was usually 3-4 x 10^7 per mouse), and seeded in Petri dishes at a density of 5 x10^5 cells per ml of Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). In preliminary dose-finding experiments the BM cells were cultured for 3 to 7 days in the presence of varying doses of recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF; Peprotech, Rocky Hill, NJ, USA) and recombinant murine interleukin-6 (rmIL-6; Peprotech), or with a combination of rmGM-CSF, rmIL-6, and recombinant murine granulocyte colony stimulating factor (rmG-CSF; Peprotech). On the basis of phenotypic and functional characteristics, the optimal protocol for

BM-MDSC generation was found to be a 3-day culture of BM cells in the presence of rmGM-CSF, rmIL-6, and rmG-CSF (10 ng/ml each).

DCs, as Ag-presenting cells (APCs), were also generated from BM of naïve wt BALB/c mice by culturing BM cells for 9 days in the presence of 40 ng/ml rmGM-CSF, as described previously [65].

These experiments were performed by the author.

4.1.4 Phenotypic analysis of mouse cells by flow cytometry

In order to assess the effect of BM-MDSCs on DC maturation, DCs were cultured alone or in the presence of BM-MDSCs for 2-3 days prior to flow cytometric measurement of the levels of MHC II and CD86 expression. Similarly, T cells were co-cultured with Ag-loaded DCs in the absence or presence of BM-MDSCs (as described below for T cell proliferation assays), and the effect of BM-MDSCs on T_{REG} cell differentiation and cytokine production was determined by flow cytometry after intracellular staining (see below).

After harvesting the cells of interest, cells were suspended in flow staining/washing buffer (PBS containing 0.05% bovine serum albumin and 0.05% sodium azide). Prior to surface staining with fluorochrome-tagged mAbs, Fc receptors were blocked with purified anti-CD16/CD32 mAb (Fc block; rat mAb, clone 2.4G2; BD Biosciences, San Diego, CA, USA) for 10 minutes at 4°C. Immunostaining was performed using fluorochrome-conjugated mAbs (obtained from BD Biosciences, eBioscience, or BioLegend, San Diego, CA, USA) against the following cell surface markers: CD11b (rat mAb, clone M1/70), Gr-1 (rat mAb, clone RB6-8C5), Ly6C (rat mAb, clone HK1.4), Ly6G (rat mAb, clone 1A8), F4/80 (rat mAb, clone RM8), CD115 (rat mAb, clone AFS98), CD80 (hamster mAb, clone 16-10A1), CD11c (hamster mAb, clone N418), MHC II ($I\text{-}A^d/I\text{-}E^d$) (rat mAb, clone M5/114.15.2), CD86 (rat mAb, clone GL-1), CD3 (hamster mAb, clone 145-2C11), CD4 (rat mAb, clone GK1.5), and CD25 (rat mAb, clone PC61). Separate cell aliquots were stained with fluorochrome-labeled isotype-matched rat or hamster control IgGs. For detection of Tregs, cells were first stained for CD4 and CD25, permeabilized, and stained for intracellular FoxP3 using a mouse FoxP3 staining kit (eBioscience). A staining protocol and a fixation/permeabilization kit (Cytofix/Cytoperm kit with GolgiStop from BD Biosciences) were employed to detect intracellular cytokines. In brief, the cells (2×10^6/ml culture medium) were first incubated with 10 ng/ml phorbol-13-myristate acetate (PMA, Sigma-Aldrich), 1 μg/ml Ionomycin

(Invitrogen, Grand Island, NY, USA), and 1 μl/ml GolgiStop (2 μM Monensin) for 4 hours. After surface staining for CD4 (rat mAb, clone GK1.5) the cells were fixed, permeabilized, and stained with fluorochrome-conjugated mAb to murine interferon gamma (IFNγ) (rat mAb, clone XMG1.2; BioLegend) or IL-10 (rat mAb, clone JES5-16E3; eBioscience). Flow cytometry was performed using a BD FACS Canto II instrument, and data were analyzed with FACS Diva software (BD Flow Cytometry Systems, San Jose, CA, USA).

These experiments were performed by the author.

4.1.5 Immunofluorescence imaging and cytospin preparations of mouse MDSCs

Occasionally, BM-MDSCs, generated from EGFP-LysM-Tg mice, which express EGFP only in cells of myeloid origin [55] were used for fluorescence imaging. In brief, after BM-MDSCs were immunostained with fluorochrome-labeled mAbs to Ly6G and Ly6C (specified above), a small aliquot of cell suspension was placed in a 0.5 mm-deep imaging chamber (Invitrogen). The cells were visualized using a Prairie Ultima two-photon microscope system (Prairie Technologies, Middleton, WI, USA), and images were created with Imaris software (Bitplane, South Windsor, CT, USA) as described previously [65, 67].

For analysis of cell morphology, BM-MDSCs or SF cells were spun onto glass slides, air dried, and stained with Wright-Giemsa solution (Sigma-Aldrich). Cytospin preparations were viewed and photographed as described for joint histology.

These experiments were performed by the author.

4.1.6 Measurement of GM-CSF, IL-6, and G-CSF levels in mouse serum and SF

Concentrations of GM-CSF, IL-6, and G-CSF in serum and cell-free SF samples of arthritic mice were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits from Peprotech. Serially diluted (1:50-1:400) serum and SF samples and the appropriate standards were incubated in plates coated with anti-GM-CSF, anti-IL-6, or anti-G-CSF Abs, and plate-bound material was detected according to the manufacturer's instructions. Absorbance at 450 nm was read by a Synergy 2 ELISA reader (BioTek Instruments, Winooski, VT, USA).

These experiments were performed by the author.

4.1.7 Purification of T cells and depletion of Ly6C^{hi} monocytic MDSCs

T cells were purified from the spleens of naive PG-TCR-Tg BALB/c mice by negative selection using an EasySep Mouse T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada). The purity of T cells, verified by flow cytometry, was greater than 95% in all cases.

Depletion of Ly6C^{hi} (monocytic) cells from the total BM-MDSC population was carried out using an EasySep Biotin Selection Kit (StemCell Technologies). Unwanted cells were targeted with a biotinylated mAb against Ly6C (rat mAb, clone HK1.4), followed by immunomagnetic depletion of the mAb-tagged cells. This resulted in the removal of essentially all Ly6C^{hi} (but not Ly6C$^{int/lo}$Ly6G^{hi}) BM-MDSCs, as confirmed by flow cytometry.

These experiments were performed by the author.

4.1.8 Assays for determining MDSC-mediated suppression of T cell proliferation

For assessment of suppression of Ag-dependent T cell proliferation first the DCs were cultured overnight with the recombinant G1 domain of human PG (rhG1; 7.5 μg/ml) [72] as Ag in the absence or presence of BM-MDSCs, Ly6C^{hi} cell-depleted BM-MDSCs, or SF cells (as suppressors) in quadruplicate wells of 96-well plates. T cells purified from the spleens of naive PG-TCR-Tg mice were added and co-cultured for 5 days at a T cell : DC : suppressor cell ratio of 1 : 0.3 : 1. Background controls included the following: T cells and DCs co-cultured without Ag (rhG1) and each suppressor population cultured alone for the same length of time (5 days). The cells were pulsed with [^{3}H]thymidine (Perkin Elmer, Waltham, MA, USA) at 1 μCi/well for the last 18 hours of culture, and isotope incorporation (counts per minute: cpm) was measured in a MicroBeta scincillation counter (Perkin Elmer).

In order to assess Ag-independent suppression of T cell proliferation, 96-well plates were first coated with purified mAbs against CD3 (hamster mAb, clone 145-2C11) and CD28 (hamster mAb, clone 37.51) (1 μg of each per well in 100 μl sterile sodium carbonate buffer, pH 9.6). T cells were added to the coated wells alone, or with an equal number of BM-MDSCs, Ly6C^{hi} cell-depleted BM-MDSCs, or SF cells as suppressors. Background controls were T cells cultured in uncoated wells and suppressors cultured in anti-CD3/CD28-coated wells. T cell proliferation was measured on day 4 of culture as described above.

In all cases, the results of proliferation assays were expressed as percent suppression after correction for backround proliferation using the following equation [65]:

% suppression = 100 – [(cpm with suppressors/cpm without suppressors) x 100].

To inhibit MDSC-mediated suppression, the following inhibitors of MDSC products were added to the co-cultures of T cells, Ag-loaded DCs, and BM-MDSCs (or anti-CD3/CD28-stimulated T cells and BM-MDSCs): N^{ω}-hydroxy-nor-arginine (nor-NOHA; 0.5 mM), an inhibitor of arginase 1; N^{G}-monomethyl-L-arginine acetate (L-NMMA; 0.5 mM) and 1400W (0.1mM), inhibitors of inducible nitric oxide synthase (iNOS); Z-VAD-FMK (0.1 mM), an inhibitor of caspases and caspase-mediated apoptosis (all inhibitors were purchased from Calbiochem, Gibbstown, NJ, USA); or the ROS scavenger catalase (1,000 U/ml, Sigma-Aldrich). Cell proliferation results were expressed as % suppression in the presence and absence of each inhibitor.

The author participated in all the experiments but adding the [^{3}H]thymidine to the cultures was done by Katalin Mikecz or Beata Tryniszewska. Katalin Mikecz occasionally (20-30%) also contributed in the tissue cultures.

4.1.9 Reverse transcription-polymerase chain reaction (RT-PCR)

As described in our previous study [65] the transcript for murine iNOS (*Nos2*) was expressed at much lower levels in spleen cells than SF cells obtained from arthritic mice. Therefore, we used spleen cells as a reference control to determine if the *Nos2* gene was also upregulated in BM-MDSCs. Total RNA was isolated from BM-MDSCs and control spleen cells using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was synthesized employing a SuperScript First Strand kit (Invitrogen), and PCR was performed using HotStart Taq Plus enzyme (Qiagen, Carlsbad, CA, USA) in 35 cycles (95°C for 20 sec, 57°C for 30 sec, and 72°C for 45 sec) with a final extension at 72°C for 10 min in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). A murine *Nos2*-specific primer pair (*Nos2* forward 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3', and *Nos2* reverse 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3') was used to detect the *Nos2* transcript, and an *Actb* gene-specific primer pair (*Actb* forward 5'-TGGCTCCTAGCACCATGAAGATCA-3' and *Actb* reverse 5'-ATCGTACTCCTGCTTGCTGATCCA-3') served for detection of the housekeeping gene encoding β-actin. After amplification, samples were loaded onto 1.5% agarose gels.

The author collected the samples for this experiment but all the PCRs were performed by Tibor A. Rauch.

4.1.10 Western blot

BM-MDSCs and control spleen cells were lysed in cold RIPA buffer containing a Halt protease inhibitor mixture (Pierce/Thermo Fisher, Rockford, IL, USA), and the protein content was determined using the bichinconic acid assay (Pierce). Proteins from cell lysates (20 μg protein each) were loaded onto and resolved in 7.5% SDS-PAGE gels (Bio-Rad) under reducing conditions. The proteins were then transferred to nitrocellulose membranes. The membranes were blotted with an anti-mouse iNOS mAb (mouse mAb; Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:500 dilution. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 (Invitrogen) was used as a secondary Ab at a 1:10,000 dilution. The protein bands were visualized using the enhanced chemiluminescence method (Amersham/GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were stripped and re-probed with a HRP-conjugated mAb to β-actin (mouse mAb, clone mAbcam 8226; Abcam, Cambridge, MA, USA) at a 1:5,000 dilution to ensure equal sample loading.

The author collected the samples for this experiment but all the Western blots were performed by Beata Tryniszewska.

4.1.11 Measurement of iNOS activity

To measure iNOS enzymatic activity (NO production) in the supernatants of 2-day co-cultures of murine BM-MDSCs, DCs and T cells, a nitrite/nitrate colorimetric assay was performed according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA). Supernatants of spleen cell cultures, containing the same number of cells as the co-cultures, were used as a reference. Samples were run on a BioTek microplate reader and absorbance was measured at 540 nm. A standard curve was generated using nitrate standards serially diluted between 5 μM and 35 μM. Results were expressed as total nitrate concentration (μM).

These experiments were performed by the author.

4.1.12 Induction of adoptively transferred PGIA in SCID mice and BM-MDSC transfer

Adoptive cell transfer from wt BALB/c to SCID BALB/c mice is an ideal tool for investigating the *in vivo* distribution and effects of donor cells, as the syngeneic SCID mice exhibit complete tolerance to the wt donor cells, allowing these cells (e.g., lymphocytes) to expand rapidly *in vivo* [73]. SCID BALB/c mice also develop PGIA after spleen cell transfer from arthritic donors in a more uniform and synchronous manner than wt BALB/c mice following PG immunization [73]. In order to induce adoptively transferred PGIA in SCID BALB/c mice, spleen cells from arthritic wt BALB/c donors were injected intravenously into SCID recipients (~10^7 cells/mouse). At the time of spleen cell transfer, SCID mice also received 100 μg of human PG (without adjuvant) i.p. to re-activate the donor cells *in vivo* [73, 74]. When arthritis started to develop (day 15 after the first splenocyte transfer), mice were divided into two groups (n = 10 mice/group) with mean disease scores of 2.0 and 2.05, respectively. One group received a second transfer of 10^7 splenocytes with 100 μg of human PG i.p., and the other group was co-injected i.p. with spleen cells and BM-MDSCs (~10^7 of each cell type/mouse) together with the same dose of PG. Control mice (injected with only splenocytes and PG twice) and BM-MDSC-treated mice (also receiving BM-MDSCs with the second injection of spleen cells and PG) were examined twice a week for disease severity and scored as described for the primary form of PGIA. Mice were sacrificed on day 34 after the first cell transfer for determination of joint histopathology and PG-specific immune responses. Arthritis severity data were collected from 2 independent experiments, each having 5 mice per group (10 mice total per group).

We also carried out a separate experiment on a limited number of SCID mice (n=3) to assess the distribution of transferred BM-MDSCs in various tissues. In order to distinguish the transferred cells from the recipients' own MDSCs, BM-MDSCs were generated from EGFP-LysM-Tg BALB/c mice that express EGFP in myeloid cells [73, 74]. As described above, SCID mice injected with PG and 10^7 spleen cells from arthritic wt BALB/c mice on days 0 and 15 also received 5 x 10^6 $EGFP^+$BM-MDSCs i.p. on day 15. This amount of BM-MDSCs (half of the therapeutic dose) only weakly inhibited arthritis progression, which enabled us to detect donor cells in the SF of recipient mice. On day 34, blood, SF, BM, spleen, and joint-draining LNs were harvested from the recipient mice. The cells were immunostained for CD11b, Ly6C, and Ly6G, and the subset composition of $EGFP^+CD11b^+$ donor cells was determined by flow cytometry.

These experiments were performed by the author but occasionally (20-30%) Katalin Mikecz helped with the cell transfer process.

4.1.13 Measurement of PG-specific T cell responses and serum Abs in SCID mice

Spleens of SCID mice were harvested and splenocytes were seeded in 96-well culture plates at a density of 2×10^5 cells per well in DMEM containing 10% FBS in the presence or absence of purified human PG (25 μg/ml) as Ag in triplicate wells. Cells were cultured for 5 days, and proliferation was measured on the basis of [^{3}H]thymidine incorporation. Results were expressed as stimulation index (SI), which is a ratio of isotope incorporation (cpm) by PG-stimulated and non-stimulated cultures.

Concentrations of PG-specific Abs in the sera of SCID mice were determined by ELISA as described elsewhere [67, 71]. Briefly, MaxiSorp ELISA plates (Nunc, Denmark) were coated with human PG (0.75 μg/well) overnight. Unbound material was washed out, and the wells were blocked with 1.5% fat-free milk in PBS. Serially diluted (1:100-1:200,000) serum samples from individual mice, and internal standard samples (pooled serum from arthritic BALB/c mice, containing known amounts of PG-specific IgG1 and IgG2a) were incubated with the immobilized PG. PG-specific IgG1 and IgG2a were detected using HRP-conjugated secondary Abs (Invitrogen), followed by HRP substrate and *o*-phenylene-diamine (Sigma-Aldrich) as chromogen. Optical densities were measured at 490 nm in an ELISA reader. Data were expressed in mg/ml serum (PG-specific IgG1) or μg/ml serum (PG-specific IgG2a).

The author collected all the samples used in this experiments, two of the ELISAs were performed by Timea Ocsko.

4.2 Human studies

4.2.1 RA patients

Altogether 11 RA patients undergoing therapeutic joint fluid aspiration at two clinics (Section of Rheumatology of the Department of Internal Medicine, and Rheumatology Associates) at Rush University Medical Center participated in the study. Informed consent was obtained from each of the participants. The 11 RA patients all donated SF, and 9 of them also donated blood. The specimens (SF and peripheral blood from RA patients and peripheral

blood from a healthy volunteer) were collected through the Knee Injury and Arthritis Repository Study approved by the Institutional Review Board of Rush University Medical Center. All patients had established RA according to the 2010 ACR/EULAR classification criteria [29] and substantial joint effusions requiring therapeutic aspiration. The mean age of the RA patients (9 females and 2 males) was 50.3 years (age range: 33-61 years).

4.2.2 Analysis of cell surface marker expression and morphology of human (RA) SF cells

Cells from the SF were pelleted by centrifugation (1000 rpm for 10 min at 4^{o}C) and washed with sterile culture medium. Most of the SF samples had visible fibrin clots (formed in the syringe after joint aspiration). In these cases, clots and large cell aggregates were removed by passing the cell suspension through sterile 70 μm pore-size cell restrainer filters (BD Biosciences). The cells were then counted and used for phenotypic, morphologic, and functional analyses without further separation. The reason for not separating MDSCs, which involves antibody-based positive selection for CD11b^{+} myeloid cells followed by sorting for CD33^{+}HLA-DR^{-} MDSC-like cells, was that antibodies against CD11b (Mac-1 integrin α chain) had been reported to interfere with the function of myeloid cells including inhibition of T cell responses [75, 76]. An aliquot of SF cells was immunostained and processed for flow cytometry. Before immunostaining, Fc receptors were blocked with purified human FcR inhibitor (eBioscience) and then the cells were stained with fluorochrome-labeled monoclonal Abs (mAbs) against the following surface markers: CD11b, CD33, HLA-DR, CD14, and CD15 (from eBioscience or BioLegend). Flow cytometry was performed using a BD FACS Canto II instrument, and data were analyzed with FACS Diva software. For analysis of cell morphology, an aliquot of SF cells was spun onto glass slides, air dried, and stained with Wright-Giemsa solution (Sigma-Aldrich). Cytospin preparations were viewed and photographed using a Nikon Microphot light microscope equipped with a digital CCD camera.

4.2.3 Cell isolation from human peripheral blood and suppression assays

Venous blood was collected in heparin-containing tubes, and peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll density gradient (GE Healthcare Life Sciences) according to a standard protocol. After extensive washing, PBMCs were suspended

in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone). Viability (usually >95%) and cell counts for PBMCs as well as for SF cells were determined prior to culture. PBMCs were seeded in 96-well plates previously coated with anti-human-CD3 mAb (1 µg/well; BioLegend) at a density of 1×10^5 cells per well in DMEM containing 10% FBS in the absence or presence of autologous (unseparated) SF cells at a ratio of 1:1. Background controls included PBMCs cultured in uncoated wells, and SF cells seeded in anti-CD3-coated wells. Anti-CD28 mAb (1 µg/well; BioLegend) was added in solution to cells in all anti-CD3-coated wells. The cells (4-6 wells/condition) were cultured for 4 days, and pulsed with [^{3}H] thymidine (Perkin Elmer) at 1 µCi/well for the last 18 hours of culture. Isotope incorporation (counts per minute: cpm) into dividing cells was measured in a MicroBeta scintillation counter (Perkin Elmer). As the actual cpm values varied from patient to patient, we calculated the ratios of T cell proliferation in the presence of SF cells relative to positive control (i.e., in the absence of SF cells) after background correction.

We obtained sufficient numbers of both PBMCs and SF cells from 3 patients (RA #7, 8, and 9) to compare the effects of SF cells on anti-CD3/CD28-induced (non Ag-specific) and alloreactive (Ag-specific) proliferation of T cells side by side. To induce Ag-specific (allogeneic mixed leukocyte) response, RA PBMCs were co-cultured with allogeneic PBMCs from a HLA-mismatched healthy donor in the absence or presence of autologous SF cells at a RA PBMC:normal PBMC:RA SF cell ratio of 1:1:1. The allogeneic cells were treated for 30 min with Mitomycin C (40 µg/ml; Sigma-Aldrich) prior to co-culture to inhibit cell division, and proliferation of autologous (RA) T cells was determined on day 5 on the basis of [^{3}H]thymidine incorporation. In this case, cmp values of Mitomycin C-treated allogeneic PBMCs (cultured alone) were included in the background controls.

Isotope incorporation (cpm values) by the cells of this group of 3 RA patients was reasonably similar when the cells were stimulated with anti-CD3/CD28 or with allogeneic PBMCs or cultured alone. This allowed us to compare the background-corrected cpm values as well as the proliferation ratios under the two different conditions of *in vitro* stimulation.

The author designed and performed most of the human experiments, analyzed and interpreted the data. The author participated in all experiments, in collecting and processing the samples and obtaining the consent forms. However, obtaining the human samples was a joint team effort as follows: Carla R. Scanzello helped with the organization of the collection of human specimens, Robert S. Katz provided RA specimens and patient information, Anjali Nair helped with the obtaining of the informed consent from RA patients. Andras Vida

contributed in about 30% of the flow cytometry of human samples. Timea Ocsko participated in the T cell proliferation assays from 10-20%. Katalin Mikecz contributed in the study design and statistics.

4.3 Statistical analysis

Descriptive statistics was employed to determine the means ± SEM and 95% confidence intervals (95% CI) unless noted otherwise. Statistical analysis was performed using GraphPad Prism 6 program (GraphPad Software, La Jolla, CA, USA). For the animal studies, the parametric Student's t test or the non-parametric Mann-Whitney U test was employed to compare two groups of data. Multiple comparisons were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Data resulting from repeat measurements over time were analyzed using two-way repeated measures analysis of variance. For the human studies, paired t test and Wilcoxon matched-pairs signed rank test were used (as appropriate) to detect statistically significant ($p < 0.05$) differences in the cpm values and in the proliferation ratios of RA PBMCs under the different culture conditions. In all cases, P values of less than 0.05 were accepted as statistically significant.

The author participated in collecting and interpreting the data along with Katalin Mikecz who contributed greatly in the statistical analysis.

5 RESULTS

5.1 Murine BM cells cultured in the presence of G-CSF, GM-CSF, and IL-6 give rise to a cell population resembling SF-MDSCs

One of the main goals of this study was to establish a culture method by which BM cells can be enriched in myeloid cells resembling SF-MDSCs in both their phenotype and function. We chose BM because it is the body's largest reservoir of myeloid precursors from which large numbers of MDSCs can be generated under appropriate conditions. GM-CSF is essential for the survival and suppressor activity of MDSCs [77] and one study reported successful generation of MDSCs from human blood in 7 days with a combination of GM-CSF and IL-6 [53] factors that are also present in the SF of RA patients [78]. In preliminary experiments, we sought to determine whether BM cells cultured for 3 to 7 days in the presence GM-CSF and IL-6 acquire an SF-MDSC-like phenotype. Although the BM culture became enriched in $CD11b^+$ cells under this condition as determined by flow cytometry, unlike SF-MDSCs, only a small proportion of these myeloid cells expressed Ly6G, a marker of granulocytic MDSCs (data not shown). We added G-CSF as a booster of the granulocytic lineage to the BM culture, which resulted in the rise of cell populations expressing Ly6G alone, or co-expressing Ly6G (at high levels) with low-to-intermediate levels of the monocytic MDSC marker Ly6C (*Fig. 1A*, and *Fig. 1C* [left panel]). This overall phenotype was achieved in 3 days of culture in the presence of GM-CSF, IL-6, and G-CSF (10 ng/ml each); longer culture or higher doses of G-CSF did not result in increases in $Ly6G^+$ or double $Ly6C^+Ly6G^+$ cells (data not shown). In comparison with $CD11b^+$ SF cells (*Fig. 1B* and *1D*), BM-MDSCs contained fewer double $Ly6C^+Ly6G^+$ cells and higher proportions of subsets expressing only one of these markers (*Fig. 1A* and *1C*). However, cells co-expressing Ly6C and Ly6G clearly represented a dominant population in both the BM-MDSC cultures and freshly harvested SF (*Fig. 1A-D*). Our choice of the combination of growth factors to generate SF-MDSC-like cells from BM was supported by the finding that SF from mice with PGIA contained high amounts of GM-CSF and G-CSF, and detectable amounts of IL-6. In each case, the SF concentrations of these factors exceeded the serum levels (Table 5).

Table 5. Concentrations of GM-CSF, IL-6, and G-CSF in synovial fluid (SF) and serum samples from arthritic (PGIA) mice[a]

	GM-CSF (ng/ml)	IL-6 (ng/ml)	G-CSF (ng/ml)
SF #1	201.79	3.71	5.16
SF #2	706.00	5.07	122.73
SF #3	654.77	3.54	71.38
SF #4	299.40	3.50	50.64
SF #5	200.39	1.91	10.67
SF Mean ± SEM	**417.47 ± 111.13**	**3.54 ± 0.50**	**52.12 ± 21.54**
Serum #1	11.76	0.63	5.78
Serum #2	28.80	1.42	7.58
Serum #3	8.61	0.54	3.72
Serum #4	7.07	0.44	4.79
Serum #5	12.31	0.39	5.22
Serum Mean ± SEM	**13.71 ± 3.90**	**0.68 ± 0.19**	**5.41 ± 0.64**
***P* value[b] SF vs serum**	**0.008**	**0.003**	**0.056**

[a]Each SF and serum sample was pooled from at least 3 mice.

[b]*P* value was calculated using the Student's t test or the Mann-Whitney U test.

Immunofluorescence staining of BM-MDSC-like cells generated from EGFP-LysM mice (cultured for 3 days as described above), followed by imaging with TPM demonstrated that the majority of myeloid ($EGFP^+$) cells expressed either Ly6G or Ly6C, or both markers (*Fig. 1C*, middle panel). Both polymorphonuclear granulocyte (neutrophil)-like cells (*Fig. 1C*, right panel: arrows) and large precursor-like cells (*Fig. 1C*, right panel: arrowheads) were seen in the cytospin preparations of such cells.

Overall, the flow cytometry profile and morphology of BM-MDSC-like cells (*Fig. 1C*) demonstrated greater heterogeneity than those of fresh SF cells (*Fig. 1D*), suggesting that in addition to the dominant population of double-positive $Ly6G^{hi}Ly6C^{int/lo}$ cells (also present in SF), BM-MDSC cultures contained a variety of immature myeloid cells with intermediate phenotypes.

Ly6G is highly expressed by both mature neutrophils and granulocytic MDSCs in mice [65]. No additional surface markers are available to distinguish between these two types of cells. On the other hand, among monocytic cells, classical (or "inflammatory") monocytes are characterized by high expression of Ly6C, whereas non-classical (also termed "patrolling" or "anti-inflammatory" monocytes/macrophages) express Ly6C at low levels.

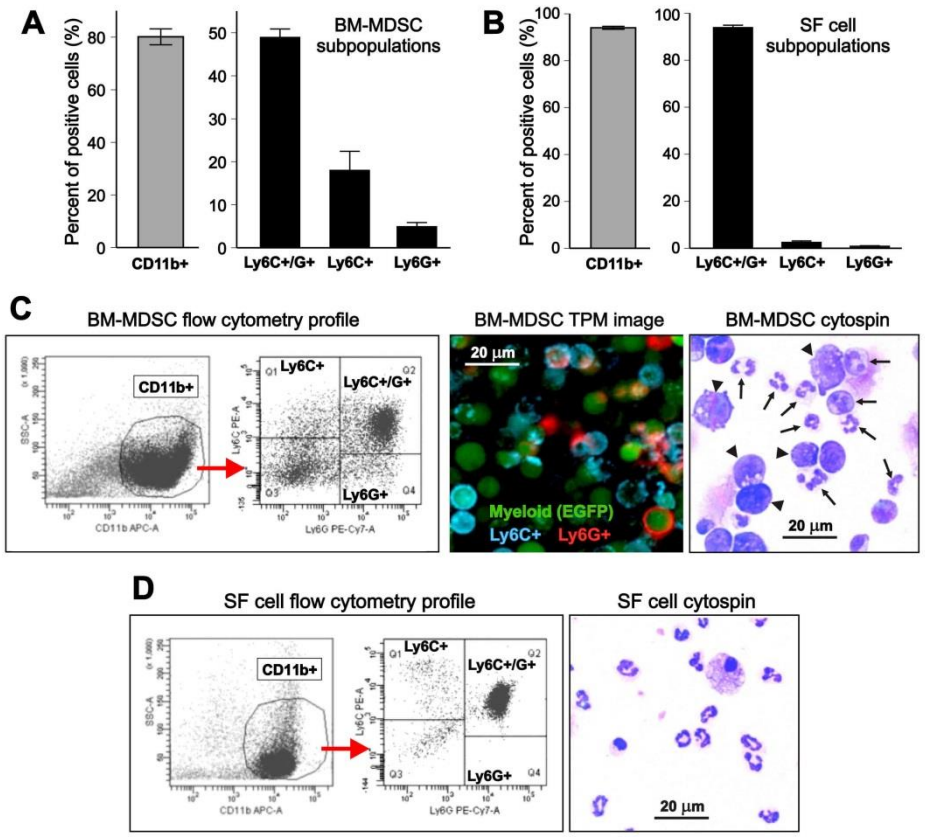

Figure 1. Phenotype and morphology of myeloid-derived suppressor cell (MDSC)-like cells generated *in vitro* from murine bone marrow (BM) in comparison with synovial fluid (SF) cells.
(**A**) Phenotype of MDSCs arising from growth factor-cytokine treated BM cell cultures as determined by flow cytometry. BM cells were cultured in the presence of GM-CSF, IL-6, and G-CSF (10 ng/ml each). On day 3, cells were immunostained for CD11b, Ly6C, and Ly6G. Approximately 80% of the cells expressed the common myeloid marker CD11b (gray bar). Most cells co-expressed Ly6C (marker of the "monocytic" subset) and Ly6G (marker of the "granulocytic" subset), but cells expressing only one marker were also present (black bars). The results are the means ± SEM of 7 independent BM cultures. (**B**) In SF, the vast majority of the $CD11b^+$ myeloid population (gray bar) was found to be cells co-expressing Ly6G and Ly6C, and lower proportions of cells expressed Ly6C or Ly6G only (black bars) than in the BM-MDSC cell cultures. The results are the means ± SEM of 7 separate pools of SF cells freshly harvested from arthritic mouse joints. (**C**) Flow cytometry profile of BM-MDSCs (left panels) is shown as an example of subset identification after gating on $CD11b^+$ cells. Fluorescence image of $EGFP^+$ BM-MDSCs (middle panel) after surface staining with a blue fluorescent antibody to Ly6C and a red fluorescent antibody to Ly6G shows cells expressing one or both markers. BM for culture was obtained from an EGFP-LysM-Tg mouse expressing EGFP (green fluorescence) in myeloid cells. Imaging was performed using two-photon microscopy (TPM). Morphology of BM-MDSCs (right panel) was visualized by Wright-Giemsa staining of a cytospin preparation, which shows both polymorphonuclear granulocyte (neutrophil)-like cells (arrows) and large precursor-like cells (arrowheads). (**D**) Flow cytometry profile (left) and morphology (right) of SF cells harvested from the arthritic joints of mice with PGIA. While the $CD11b^+$ myeloid population is large in both the BM-MDSC culture and arthritic SF, and is dominated by Ly6C/Ly6G double positive cells in both samples (analyzed simultaneously), BM-MDSCs show greater heterogeneity in morphology than SF cells.

We used additional mAbs against monocyte/macrophage markers, including F4/80, CD115, and CD80 to identify distinct subsets within the two monocytic cell categories.

However, we found that only a few percent of cultured BM-MDSCs or freshly harvested SF cells expressed F4/80 and CD80, and less than 1% of them was $CD115^+$. The highest proportions of $F4/80^+$ and $CD80^+$ cells were detected within the $Ly6C^{lo/-}$ population among BM-MDSCs (4-5%) and in the $Ly6C^{hi/int}$ population among SF cells (0.5-2%). $CD115^+$ cells represented 0.2% of both $Ly6C^{hi/int}$ and $Ly6C^{lo/-}$ BM-MDSCs, and 0.7% of $Ly6C^{lo/-}$ SF cells (data not shown).

5.2 BM-MDSCs have the ability to suppress both Ag/DC-dependent and -independent proliferation of T cells *in vitro*

In order to study the effect of BM-MDSC-like cells on Ag-specific T cell proliferation, we cultured Ag (rhG1)-loaded DCs with T cells isolated from the spleens of naive PG-TCR-Tg mice in the presence or absence of BM-MDSCs as suppressors. Additional "suppressors" (as comparators) were SF cells, and BM-MDSCs depleted in $Ly6C^{hi}$ cells. Ag-dependent T cell proliferation was dramatically reduced in the presence of BM-MDSCs, i.e., BM-MDSC-mediated suppression reached nearly 100% (*Fig. 2A*, red bar). Compared with SF cells (*Fig. 2A*, gray bar) BM-MDSCs were equally potent in suppressing T cell proliferation. As also reported for SF cells [65], depletion of the $Ly6C^{hi}$ monocytic subset from the BM-MDSCs (*Fig. 2A*, black bar) did not reduce their suppressive capacity. BM-MDSC-mediated suppression of Ag-specific T cell proliferation was accompanied by significant decreases in the percentage of $CD4^+$ T helper (T_H) cells containing intracellular cytokines (IFNγ in T_H1 and IL-10 in T_H2 cells) as well as in the percentage of T_{REG}s ($CD4^+CD25^+$ cells containing FoxP3) *(Fig. 2B*).

Since we found previously that SF-MDSCs from arthritic mice suppressed the maturation and Ag presenting capacity of DCs [65], we investigated the effect of BM-MDSCs on the expression levels of DC maturation markers MHCII and CD86. However, we could not detect significant changes in the expression level of either marker in DCs upon co-culture with BM-MDSCs (data not shown). Since BM-MDSCs failed to decrease the surface expression of these molecules by the DCs, this experiment also suggested that the observed suppression of the proliferation and cytokine/FoxP3 content of T cells was not due to release of cytotoxic substances from the BM-MDSCs.

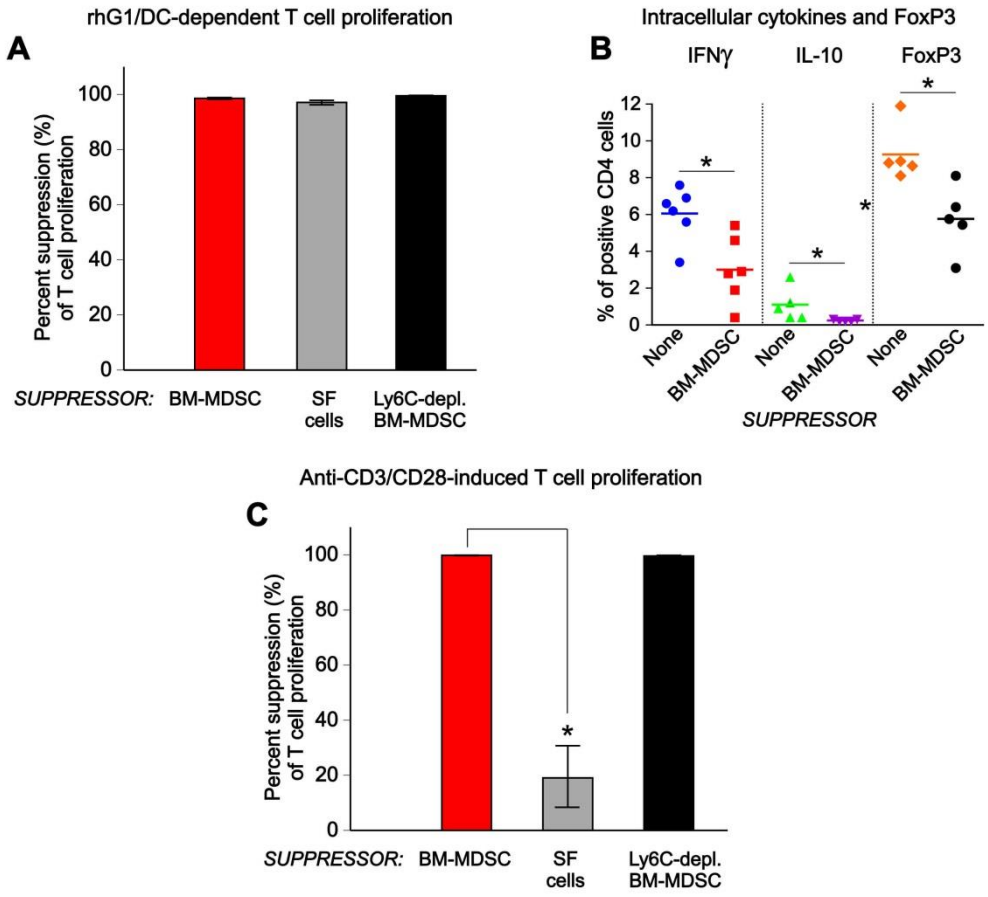

Figure 2. Suppression of antigen (Ag)-specific and non-specific T cell responses by BM-MDSCs.
(**A**) T cells, purified from the spleens of mice expressing a PG-specific T cell receptor transgene (PG-TCR-Tg) were cultured for 5 days with dendritic cells (DCs) loaded with recombinant G1 domain of human PG (rhG1) in the absence or presence of the following "suppressors": BM-MDSCs (red bar), arthritic SF cells (gray bar), or Ly6C^{hi} (monocytic) cell-depleted BM-MDSCs (black bar). The ability of suppressors to inhibit Ag (rhG1)-specific T cell proliferation (which is also dependent on Ag presentation by DCs) was assessed on the basis of inhibition of [^{3}H]thymidine incorporation by the T cells. Percent suppression was calculated as described in the Methods. All suppressors exhibited robust inhibition of T cell proliferation. The results shown are from 5 independent experiments. (**B**) T cells from PG-TCR-Tg mice were cultured for 2 days with rhG1-loaded DCs and BM-MDSCs as described for panel A. The percent of CD4^{+} T cells containing IFNγ, IL-10, or FoxP3 (CD4^{+}CD25^{+}FoxP3^{+} T regulatory cells, Tregs) was determined by flow cytometry. The results shown are the individual values (n=5-6) and the means. On average, the percentages of IFNγ^{+} cells, IL-10^{+} cells, and Tregs were lower in the presence of BM-MDSCs (*p< 0.001, 0.001, and 0.05, respectively; Mann-Whitney U test) than in their absence (None). (**C**) T cells from PG-TCR-Tg mice were cultured in anti-CD3/CD28-coated plates for 4 days in the absence or presence of the listed suppressors. Percent suppression was calculated and results expressed as described for panel A. Non-depleted BM-MDSCs and BM-MDSCs depleted in Ly6C^{hi} cells were equally potent in suppressing anti-CD3/CD28-induced T cell proliferation, while arthritic SF cells exhibited much weaker inhibition (*p<0.01, n=5; Kruskal-Wallis test followed by Dunn's multiple comparisons test) in this induction system.

In order to determine whether the suppressive effect of BM-MDSCs on T cell proliferation was Ag-dependent (for which the presence of DCs was required) or Ag-independent, we stimulated the PG-TCR-Tg T cells with anti-CD3 and anti-CD28 mAbs in the presence or absence of BM-MDSCs. In this Ag/DC-independent system, BM-MDSCs

also exhibited potent suppressor activity (*Fig. 2C*, red bar), whereas suppression by SF cells was very weak (*Fig. 2C*, gray bar), consistent with our previous report [65]. As expected, depletion of $Ly6C^{hi}$ cells did not reduce the capacity of BM-MDSCs to suppress the anti-CD3/CD28-induced proliferation of T cells (*Fig. 2C*, black bar).

5.3 Suppressive effects of BM-MDSCs on T cells can be reversed by iNOS inhibitors *in vitro*

In order to reveal the possible mechanism of the suppressive activity of the BM-MDSCs, we repeated the Ag-dependent and Ag-independent T cell proliferation assays with and without various inhibitors of MDSC-related effector molecules such as arginase 1 (nor-NOHA), iNOS (L-NMMA and the more selective 1400W), and ROS (catalase). A caspase (apoptosis) inhibitor (Z-VAD-FMK) was used as a MDSC-unrelated control. Both Ag (rhG1)- and anti-CD3/CD28-induced T cell proliferation remained suppressed in the presence of the arginase 1 inhibitor, the ROS scavenger, or the caspase inhibitor (*Fig. 3A and 3B*). However, BM-MDSCs lost much of their ability to suppress T cell proliferation in both induction systems in the presence of the iNOS inhibitors (*Fig. 3),* suggesting that the main MDSC product mediating T cell suppression was NO.

5.4 BM-MDSCs exhibit upregulated iNOS expression and elevated NO production

In order to corroborate the results of T cell proliferation assays indicating a role for NO in the suppressor activity of BM-MDSCs, we performed RT-PCR to assess expression of iNOS (*Nos2*) mRNA in BM-MDSCs in comparison with spleen cells harvested from arthritic mice [65]. BM-MDSCs demonstrated significant up-regulation of *Nos2* mRNA as compared with spleen cells (*Fig. 4A*), while the housekeeping gene (*Actb*, encoding β-actin) was expressed at equal levels. The results of Western blot were consistent with the results of RT-PCR, showing a large amount of iNOS protein (~130 kDa) in BM-MDSCs, but not in spleen cells (*Fig. 4B*).

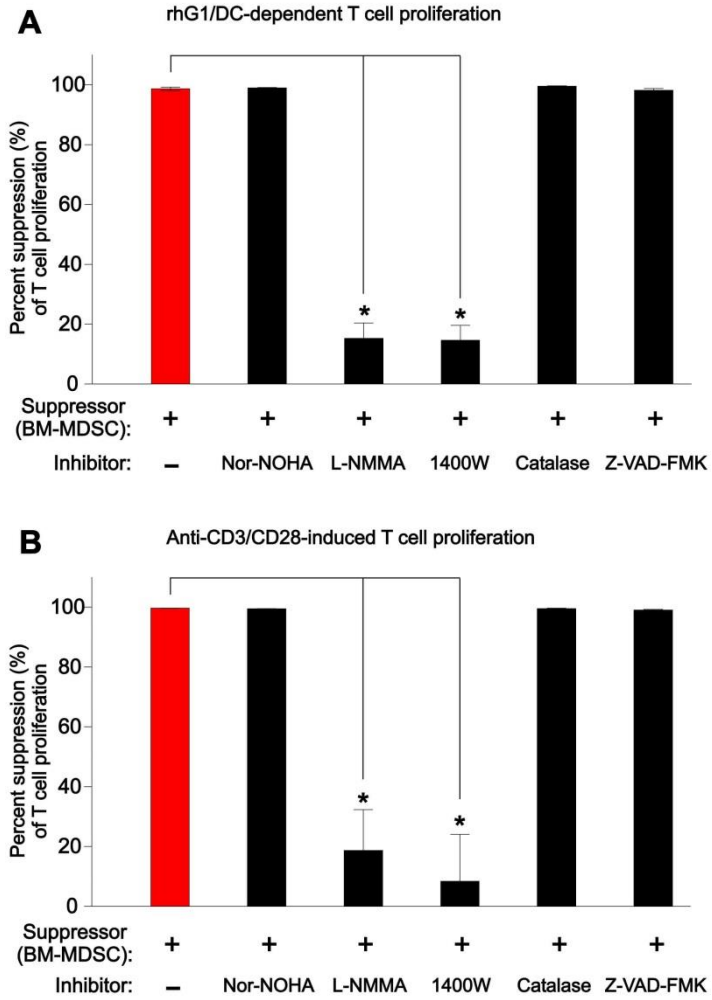

Figure 3. Reversal of the suppressive effect of BM-MDSCs on T cell proliferation by inhibitors of inducible nitric oxide synthase (iNOS).
Various inhibitors of MDSC effector molecules, including the arginase 1 inhibitor nor-NOHA, iNOS inhibitors L-NMMA and 1400W, the reactive oxygen species (ROS) scavenger catalase, and the caspase/apoptosis inhibitor Z-VAD-FMK, were used to inhibit the BM-MDSC-mediated suppression of (**A)** Ag (rhG1)-induced/DC-dependent and (**B**) anti-CD3/CD28-induced proliferation of PG-TCR-Tg T cells. The results (compiled from 2 independent series of experiments, each with 2 co-cultures) are expressed as percent suppression of T cell proliferation in the presence (black bars) or absence (red bar) of inhibitors. While suppression of T cell proliferation in both induction systems was significantly reversed by the iNOS inhibitors L-NMMA and 1400W (*$p<0.0001$ in all cases; Kruskal-Wallis test followed by Dunn's multiple comparisons test), none of the other inhibitors had a significant effect on BM-MDSC-mediated suppression of T cells.

The enzymatic activity of iNOS was assessed by measuring nitrite/nitrate concentrations (as indicators of NO production) in supernatants of BM-MDSCs (cultured in the presence of DCs and rhG1 with or without T cells) and spleen cell cultures. Consistent with the iNOS expression data, much higher levels of NO were detected in the supernatants of BM-MDSCs-containing cultures (*Fig. 4C*, orange bar) than in those of spleen cell cultures (*Fig. 4C*, turquoise bar).

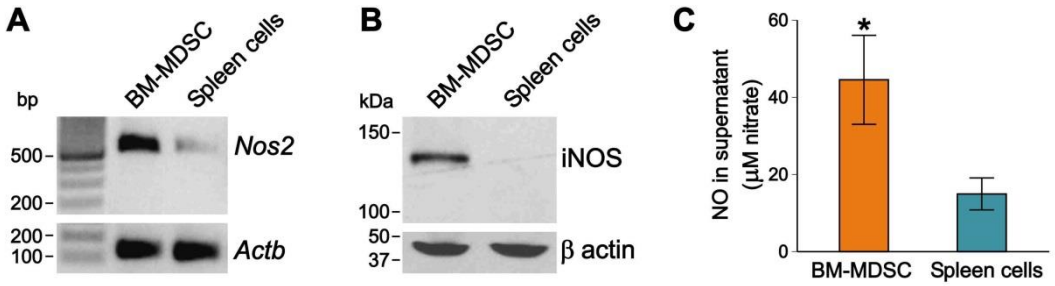

Figure 4. Expression and activity of iNOS in BM-MDSCs.
(**A**) Comparison of murine iNOS (*Nos2*) transcript levels in BM-MDSCs and spleen cells revealed that iNOS mRNA was upregulated in BM-MDSCs. The housekeeping gene (*Actb*, encoding β-actin) was expressed at equal levels. Results of one of 2 replicate experiments (with similar results) are shown. (**B**) Western blot using an antibody against murine iNOS demonstrated the presence of iNOS protein in BM-MDSCs, but not in spleen cells. The β-actin control blot shows equal sample loading. One of 3 independent Western blots is shown. (**C**) iNOS activity was assayed on the basis of NO release into the supernatants of cultures containing BM-MDSCs (orange bar) or spleen cells (green bar), and expressed as total nitrate concentration (μM). BM-MDSC-containing cultures produced significantly higher amounts of NO than spleen cells did (*p< 0.05, n=5 cultures/cell type; Mann-Whitney U test). Molecular markers: bp, base pairs; kDa, kilodalton.

5.5 Injection of BM-MDSCs into SCID mice reduces Ag-specific immune responses and ameliorates adoptively transferred arthritis

To test whether BM-MDSCs could affect the development of arthritis, an adoptive transfer model of PGIA was employed. On day 0, spleen cells from arthritic wt BALB/c donor mice were injected with Ag (human PG) into SCID recipients. When the clinical signs of arthritis started to develop (15 days after the first injection), the SCID mice were divided into 2 groups with similar mean disease scores, and a second injection was administered. The first (control) group received only arthritic spleen cells and PG, while the second group received the same plus BM-MDSCs. Arthritis severity scores in the control group increased further (*Fig. 5A*, black line), while, in sharp contrast, the scores of SCID mice transferred with BM-MDSCs remained low until the end (day 34) of the monitoring period (*Fig. 5A*, red line). Histopathology revealed massive leukocyte infiltration and synovial hyperplasia as well as cartilage erosion in the ankle (tibio-talar) joints of control SCID mice transferred with spleen cells from arthritic donors (*Fig. 5B*, left panel). In contrast, only mild synovial hyperplasia was observed without evidence of gross inflammation or cartilage damage in the ankle joints of SCID mice co-transferred with spleen cells and BM-MDSCs (*Fig. 5B*, right panel).

In order to determine whether the BM-MDSC-mediated protection from arthritis progression was associated with reduced Ag-specific T cell responses and Ab production, we

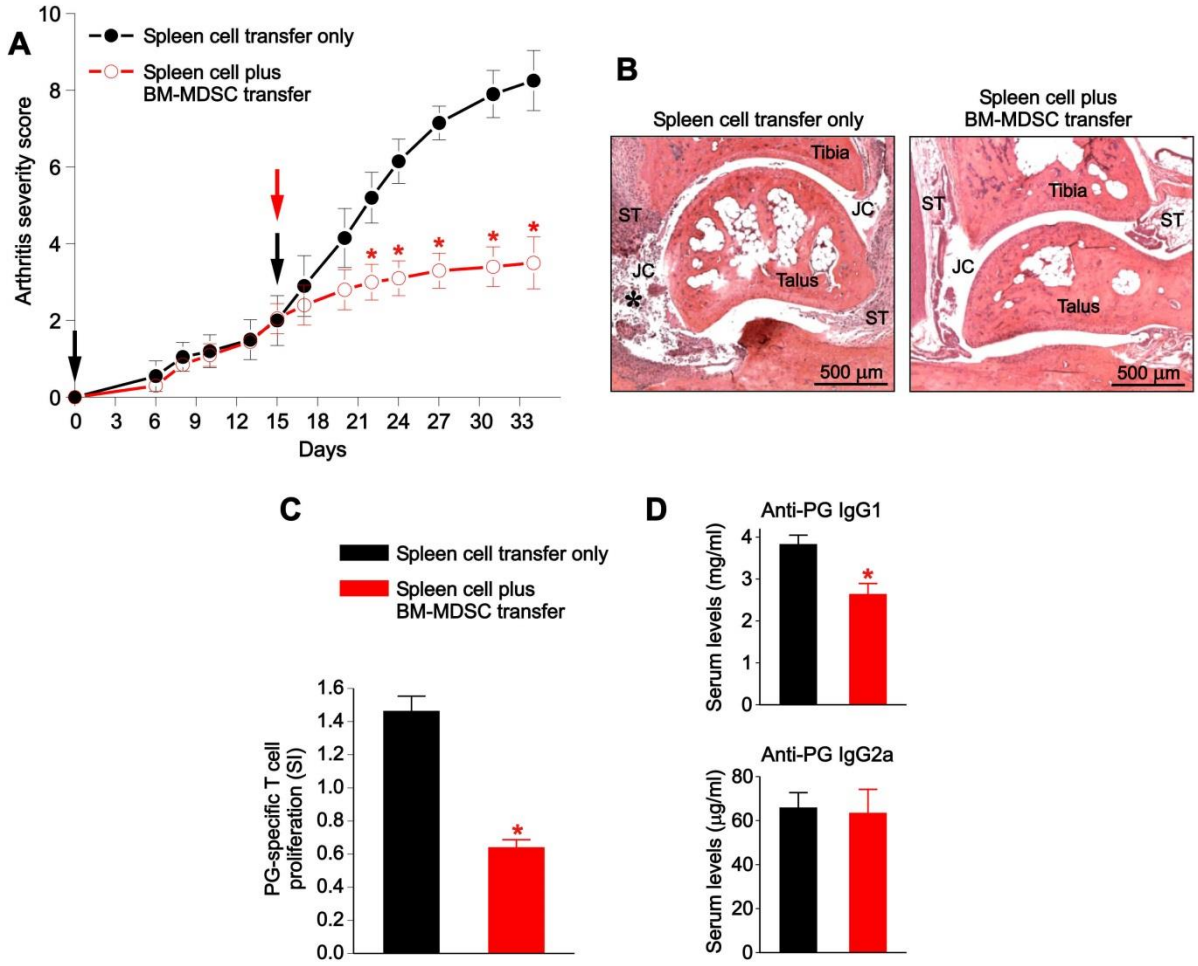

Figure 5. Effects of BM-MDSCs on arthritis severity and Ag (PG)-specific immune responses in SCID mice with PGIA.

(**A**) Effect of BM-MDSC transfer on arthritis severity. Arthritis was induced in SCID mice via 2 transfers of spleen cells (black arrows) from wild type mice with PGIA as described in the Methods. At the early phase of arthritis, one group of the SCID recipients was co-injected with BM-MDSCs (red arrow). Disease severity scores were monitored until day 34. Arthritis progressed rapidly in the control group (black line), but not in the BM-MDSC-treated group (red line) (*$p<0.05$, n=10 mice/group; two-way repeated measures analysis of variance). (**B**) Joint histopathology of control (left panel) and BM-MDSC-treated (right panel) mice on day 34. The ankle joint of the control mouse demonstrated massive leukocyte infiltration (star) in the joint cavity (JC) and synovial tissue (ST) as well as synovial hyperplasia. The articulating surfaces appeared rough due to cartilage damage. In the ankle joint of the BM-MDSC-treated mouse only mild synovial hyperplasia was seen, suggesting the resolution of initial (previous) inflammation. Representative hematoxylin-eosin-stained tissue sections from both groups are shown. (**C**) Antigen (PG)-specific T cell responses of control and BM-MDSC-treated mice. T cell responses were compared between the two groups on day 34 by measuring spleen cell proliferation in the presence or absence of PG *in vitro*. Results are expressed as stimulation index (SI), a ratio of [^{3}H]thymidine incorporation by PG-stimulated and non-stimulated cultures. The SI of the BM-MDSC-injected group (red bar) was significantly lower than the SI of the control group (black bar) (*$p< 0.0001$, n=10 mice/group; Student's t test). (**D**) Serum levels of anti-PG antibodies in the control and BM-MDSC-treated groups as determined by ELISA. The levels of IgG1 anti-PG antibodies (top) were significantly lower in the sera of BM-MDSC-injected mice than in control mice (*$p<0.01$, n=5 samples/group; Mann-Whitney U test), while the levels of IgG2a anti-PG antibodies (bottom) were similar.

compared the PG-specific T cell responses and serum IgG1 and IgG2a Ab levels in control and BM-MDSC-injected SCID mice. PG-specific T cell proliferation was significantly lower in the BM-MDSC-injected group (*Fig. 5C*). Serum levels of IgG1 isotype (but not of IgG2a isotype) anti-PG Abs were also significantly reduced in the BM-MDSC recipient group (*Fig. 5D*).

In a separate experiment, we assessed the distribution and subset composition of transferred EGFP$^+$ BM-MDSCs in various fluids and tissues (blood, SF, BM, spleens, and LNs) of SCID mice with adoptively transferred PGIA (induced as described above) 19 days after BM-MDSC injection. The donor EGFP$^+$ BM-MDSCs (injected at half of the optimal therapeutic dose) were found in considerable amounts in the blood (*Fig. 6A*), SF (*Fig. 6B*), and BM (*Fig. 6C*) of the recipient mice. The spleen (*Fig. 6D*) contained a much lower percentage of these cells, and the LNs (*Fig.* 6E) were virtually free of BM-MDSCs. In each tissue or fluid, the granulocytic subset (Ly6G^{hi}Ly6C^{int}) dominated, although small populations of monocytoid (Ly6C^{hi}Ly6G$^-$) MDSCs were also present (*Fig. 6*).

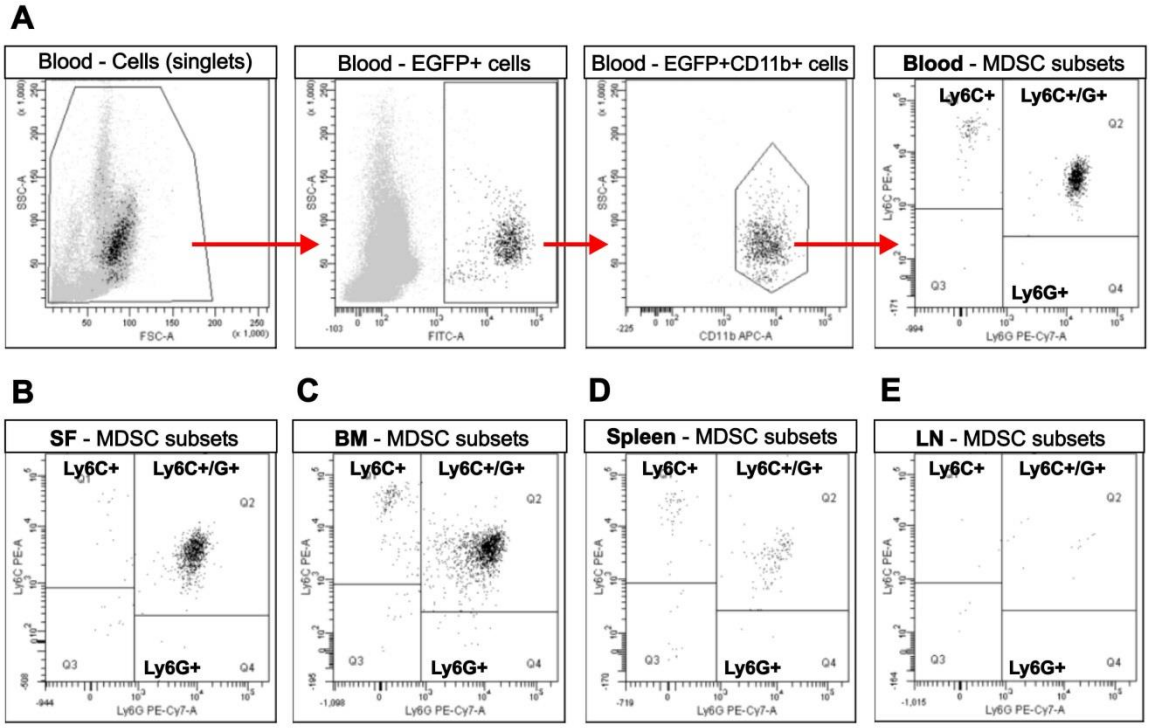

Figure 6. Tissue distribution of EGFP$^+$ BM-MDSCs injected into SCID mice with adoptively transferred PGIA.

BM-MDSCs, generated from EGFP-LysM-Tg mice (expressing EGFP in myeloid cells only) were co-injected with arthritic spleen cells into SCID mice at the early phase of adoptively transferred PGIA, as described in the Methods. To assess the tissue distribution of fluorescent donor cells, 19 days after the co-transfer of the cells (**A**) peripheral blood, (**B**) synovial fluid (SF), (**C**) BM, (**D**) spleen, and (**E**) joint-draining lymph nodes (LN) were harvested from the SCID recipients, immunostained, and subjected to flow cytometry. The gating strategy, as demonstrated on the blood cells (top panels), involved gating first on single cells, then on EGFP$^+$ cells, followed by gating on the CD11b$^+$ myeloid population (red arrows). Subset composition of EGFP$^+$CD11b$^+$ cells was determined on the basis of Ly6C and Ly6G expression. Peripheral blood, SF, and BM contained very well

detectable populations of $Ly6C^{int}Ly6G^{hi}$ (granulocytic) cells and much smaller populations of $Ly6C^{hi}Ly6G^{-}$ (monocytic) cells. Cells belonging to either subset were less frequent in the spleen, and nearly undetectable in the LNs. Representative flow cytometry dot plots of cells from 1 of 3 mice (except for SF, which was pooled from all of the 3 mice) are shown.

5.6 Cells with phenotype and morphology resembling MDSCs are present in the SF of RA patients

Screening for MDSC-like cells was carried out by flow cytometry using SF samples collected from 11 RA patients. We employed a combination of mAbs to MDSC cell surface markers including the common myeloid marker CD11b, the "immature" myeloid cell marker CD33, MHC II (HLA-DR), the monocytic MDSC subset marker CD14, and the granulocytic subset marker CD15 [45-47]. As demonstrated by a representative sample, $CD11b^{+}CD33^{+}HLA\text{-}DR^{lo/-}CD14^{-}CD15^{+}$ granulocytic MDSC-like cells were the predominant cell type in RA SF, but a small population of the $CD11b^{+}CD33^{+}HLA\text{-}DR^{lo/-}CD14^{+}CD15^{-}$ monocytic subset was also present (*Fig. 7A*).

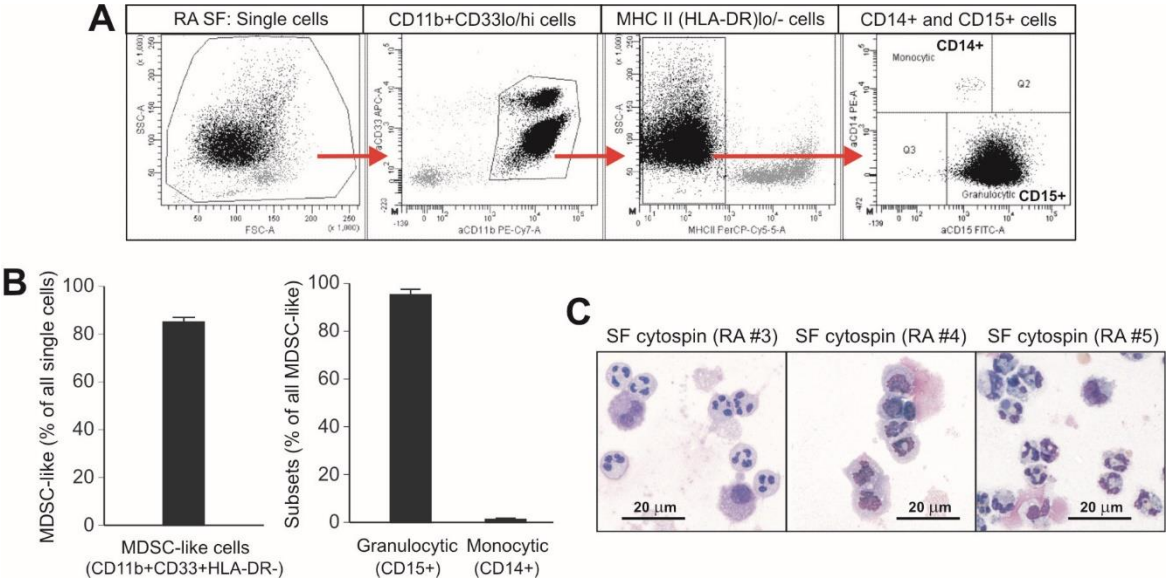

Figure 7. Presence of cells with myeloid-derived suppressor cell (MDSC)-like phenotype and morphology in synovial fluid (SF) from rheumatoid arthritis (RA) patients.
(**A**) Flow cytometry profile of RA SF cells using a combination of antibodies against the common myeloid marker CD11b, the "immature" myeloid cell marker CD33, MHC class II (HLA-DR), the monocytic subset marker CD14, and the granulocytic subset marker CD15 (gating strategy is indicated by red arrows). The example shown (1 of 11 RA SF samples with similar profiles) demonstrates the dominance of $CD11b^{+}CD33^{lo/hi}HLA\text{-}DR^{lo/-}CD14^{-}CD15^{+}$ (granulocytic) MDSC-like cells in RA SF. (**B**) Using the same gating strategy on the 11 RA SF samples, the mean frequency of MDSC-like myeloid cells was 85.03% (range: 76.1-97.9%) among the SF cells (left-side graph). The granulocytic subset represented 95.2% (range: 72.9-99.7%) and the monocytic subset represented 1.3% (range: 0.1-5.3%) of the MDSC-like SF cell population (bars in right-side graph). The data shown are the means ± SEM. (**C**) The morphology of SF cells in Wright-Giemsa-stained

cytospin preparations from 3 RA patients also indicated the dominance of the polymorphonuclear granulocytic subset, although the shape of the nuclei of these neutrophil-like cells varied among the patients.

MDSC-like cells constituted ~85% of all SF cells (*Fig. 7B*, left-side graph) and ~95% of these MDSC-like cells belonged to the granulocytic subset (*Fig. 7B*, first bar in the right-side graph) in the samples of the 11 RA patients tested. Despite the very similar cell surface marker expression profile in all RA SF samples (*Fig. 7A-B*), the nuclear morphology of the cells varied among the patients, although the dominance of the polymorphonuclear (neutrophil-like) granulocytic subset was evident (*Fig. 7C*).

5.7 RA SF cells significantly suppress the anti-CD3/CD28-induced proliferation of autologous T lymphocytes

Lechner et al [53] reported that monocytic MDSCs, generated *in vitro* from human PBMCs in the presence of GM-CSF and IL-6, were able to suppress the anti-CD3/CD28-induced proliferation of autologous T cells. To determine whether the MDSC-like cells that we identified in RA SF, indeed, had suppressive properties, we stimulated T cells (present in PBMC) with anti-CD3/CD28 mAbs in the absence and presence of SF cells from the same patients. Anti-CD3/CD28-stimulated T cells in PBMC proliferated less well in the presence than in the absence of autologous SF cells as indicated by the difference in total [^{3}H]thymidine incorporation (*Fig. 8A*, condition "a" versus condition "b'"). Isotope incorporation (cpm) by unstimulated PBMCs or anti-CD3/CD28-treated SF cells (*Fig. 8A*, conditions "c" and "d", respectively, as background controls) was low, but still detectable. We tested PBMCs and SF cells from a total of 9 RA patients in the same *in vitro* system, and found that SF cells from all of these patients suppressed anti-CD3/CD28-induced cell proliferation. Since total isotope incorporation by PBMCs and PBMC-SF cell co-cultures as well as background cpm values (conditions "c" and "d") varied from patient to patient, we calculated the background-corrected proliferation ratios for the 9 patients tested. As shown in *Figure 8B*, the SF cell-mediated suppression of anti-CD3/CD28-induced T cell proliferation was statistically significant ($p = 0.0039$, 95% CI = 0.4682-0.7495).

5.8 SF cells from the same RA patients are more potent in suppressing the Ag-specific than the anti-CD3/CD28-induced proliferation of autologous T cells

We reported previously that MDSCs present in the SF of the arthritic joints of mice with PGIA potently suppressed Ag (human PG)-induced T cell proliferation, but were ineffective against anti-CD3/CD28-induced proliferation [65]. In this study, using allogeneic PBMC as a source of Ag, we directly compared the effects of RA SF cells on the anti-CD3/CD28-induced versus alloAg-induced proliferation of autologous T cells obtained from the same patients.

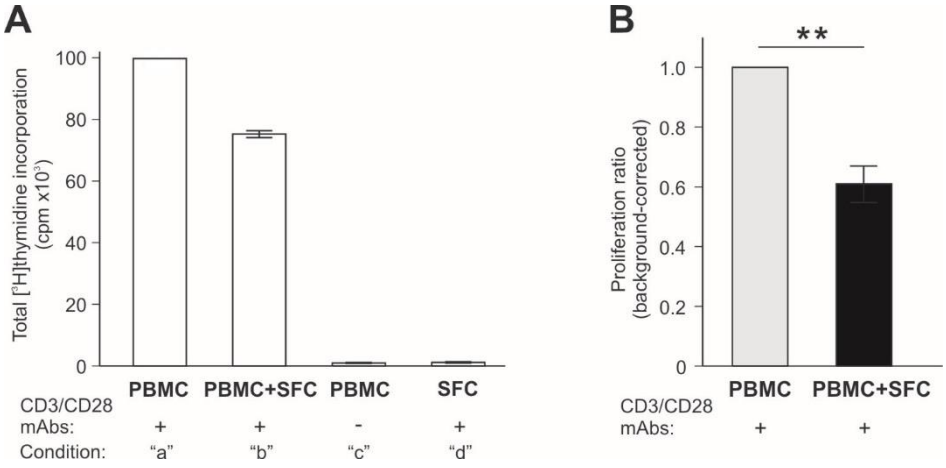

Figure 8. Suppression of anti-CD3/CD28-induced polyclonal proliferation of autologous peripheral blood T cells by RA SF cells.
(**A**) Peripheral blood mononuclear cells (PBMC) stimulated with anti-CD3/CD28 monoclonal antibodies (Abs) were cultured in the absence (condition "a") or presence ("b") of SF cells (SFC) from the same RA patient (RA #3). Background controls included PBMCs cultured without anti-CD3/CD28 Abs ("c") and SF cells cultured with anti-CD3/CD28 Abs ("d"). The results shown are the means ± SEM of isotope incorporation (counts per minute, cpm) by the proliferating cells (6 wells per condition). As the actual cpm values at all of the listed conditions varied from patient to patient, we calculated the proliferation ratio (with background correction) for each RA patient using the formula: [b - (c + d)] / (a - c), where the positive control (a - c) was set to 1. (**B**) As indicated by the proliferation ratios, the anti-CD3/CD28-induced proliferation of autologous PBMCs (light gray bar) was significantly suppressed in the presence of SF cells (black bar). The results shown are the means ± SEM of background-corrected proliferation ratios (**$p < 0.01$; Wilcoxon matched-pair signed rank test; $n = 9$ patients).

Side-by-side comparison of cell cultures of these RA patients (RA #7, 8, 9) demonstrated significant ($p = 0.0389$) but moderate activity of SF cells in inhibiting the robust proliferation of anti-CD3/CD28-stimulated autologous T cells (*Fig. 9A*), and also significant ($p = 0.0087$) and more effective suppression of the Ag-induced (and more modest) proliferation of the same T cells (*Fig. 9B*). Since the SF cell populations from the same RA

patients exhibited significantly (p = 0.0413) different degrees of suppression under the two different culture conditions (*Fig. 9C*), these results also suggested that inhibition of T cell proliferation was not simply due to cytotoxic substances released from degranulating, dying, or apoptotic SF cells upon culture.

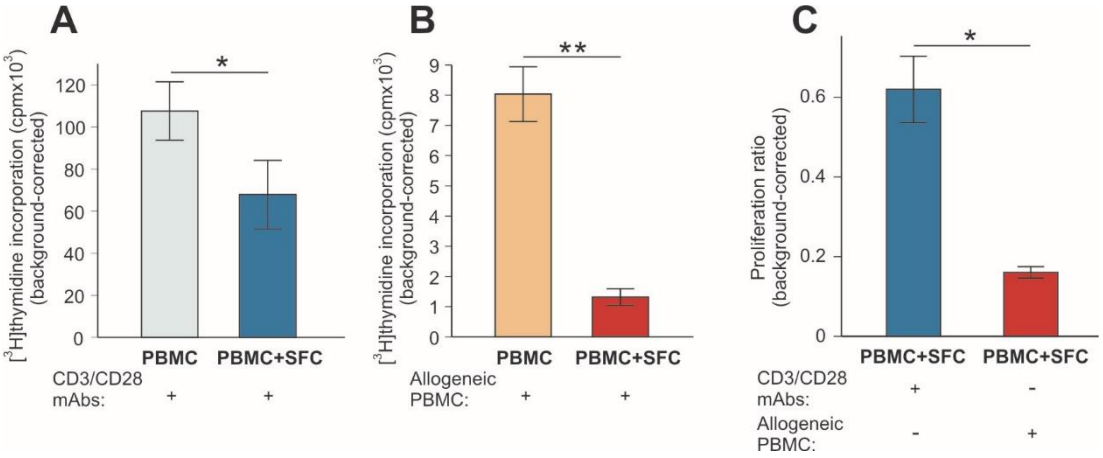

Figure 9. Comparison of the suppressive effects of RA SF cells on the anti-CD3/CD28-induced and alloantigen-induced proliferation of autologous T cells.
(**A**) Isotope incorporation (cpm) by anti-CD3/CD28 Ab-stimulated PBMCs (light blue bar) was moderately, but significantly reduced in the presence (dark blue bar) of SF cells (SFC) from 3 RA patients (RA #7, 8, 9). (**B**) Allogeneic PBMC-induced proliferation of PBMCs from the same 3 patients (orange bar) was greatly reduced in the presence of autologous SF cells (dark red bar). The data shown in panels **A** and **B** are the means ± SEM of background-corrected cpm values (4 wells per condition) (*p < 0.05, **p < 0.01; Paired t test). (**C**) Comparison of the ratios of anti-CD3/CD28-induced (dark blue bar) and allogeneic PBMC-induced (dark red bar) proliferation of RA PBMCs in the presence of autologous SFC in the 3 RA patients (cpm values shown in panels **A** and **B**) indicated that SF cells from the same patients were more efficient in suppressing the allogeneic PBMC-induced than the anti-CD3/CD28-induced proliferation of autologous T cells. The data are the means ± SEM of background-corrected proliferation ratios (*p < 0.05; Paired t test)

6 DISCUSSION

6.1 *In vitro* generated MDSCs from murine BM and their possible therapeutic role in autoimmune arthritis

MDSCs are part of the innate immune system with an exceptional ability to influence adaptive immune responses [52]. Data obtained from cancer patients and tumor-bearing animals provided the greatest amount of information on these cells [44, 45]. Lately, these cells have been identified in numerous autoimmune conditions characterized by excessive activation of adaptive immunity [46-48].

In our former paper, we demonstrated that in the PGIA model of human RA, SF contains a population of cells that resembles MDSCs [65]. The results led us to the conclusion that there is a supportive microenvironment in the inflamed joint in PGIA where myeloid cells survive and acquire a predominantly granulocytic MDSC-like phenotype. Moreover, these cells are potent suppressors of DCs and Ag-specific T lymphocytes. The expansion of pathogenic T lymphocytes and the maturation of DCs in the joint or in lymphoid organs can be suppressed by MDSCs. SF MDSCs may be able to suppress synovial inflammation. This assumption could be best confirmed by transferring MDSCs from SF to mice at the early phase of arthritis. However, MDSCs of SF origin do not proliferate in culture and the quantity of MDSCs that can be harvested from mice SF is limited [65]. This led us to seek a different opportunity to generate large amounts of MDSCs. Other authors described accumulation of MDSCs in the spleens of tumor bearing animals [79] or autoimmune disorders [47, 48]. However, in our previous study we published that in PGIA myeloid population found in the spleens was moderate in number and showed little suppressor activity [65]. Since BM contains high number of myeloid precursor cells, it seemed to be a reasonable source of cells feasible to become SF-MDSC-like cells under adequate culture conditions.

It was reported that enrichment of murine BM in immature myeloid cells was possible in cell cultures with GM-CSF, nonetheless if they added no additional factor, these cells exerted predisposition to evolve into myeloid DCs [57]. Furthermore, Lechner et al [53] published a method to generate cells with monocytic MDSC phenotype and immunosuppressive ability. In this study normal human PBMCs were cultured in the presence of IL-6 and GM-CSF. Herein we describe that mouse BM cells cultured with a mixture of GM-CSF, G-CSF and IL-6 for three days give rise to $Ly6G^{hi}Ly6C^{int/lo}$ granulocytic SF-MDSC-like cells predominantly, albeit such cultures contain modest numbers of populations

of cells with intermediate characteristic as well. Additional experiments elucidating phenotypic characteristics of the monocytic subset using mAbs to monocyte/macrophage markers, like CD80, CD115 and F4/80, did not demonstrated a distinct population of cells expressing such antigens. Nearly 5% of BM-MDSCs in the $Ly6C^{lo/-}$ subpopulation were found to be $CD11b^{+}F4/80^{+}CD80^{+}$ macrophages. Cells expressing CD115, the receptor for macrophage colony stimulating factor, were also detected in modest numbers (0.7%) within $CD11b^{+}Ly6C^{lo/-}$ SF cells. A former study revealed increased amount of $Ly6C^{hi}CD115^{+}$ osteoclast precursors in the murine BM with inflammatory arthritis [80]. These cells were described within the $CD11b^{lo/-}$ population of BM cells and showed myeloid suppressor capacity as well [80]. In our study $CD11b^{lo/-}Ly6C^{hi/int}$ populations of both BM-MDSCs and SF were virtually free of $CD115^{+}$ cells. Despite of the fact that the likelihood of $CD115^{+}$ cells in the $CD11b^{+}Ly6C^{lo/-}$ SF fraction may differentiate into mature osteoclasts cannot be ruled out completely, our data suggest that neither the SF milieu nor the BM-MDSC culture condition do not promote the generation of $CD11^{lo/-}Ly6C^{hi}CD115^{+}$ osteoclast precursor cells.

We have assumed in our former paper [65] that MDSC precursors entering the joints could obtain a phenotype of maturation-resistance in an environment of high amount of cytokines and myelopoietic growth factor. Actually herein we detected elevated levels of GM-CSF and G-CSF, and measurable levels of IL-6 in the supernatant fraction of SF of mice with PGIA. In the line with the results published by Wright et al [78] in SF and serum samples gained from RA patients, we also found much higher levels of such cytokines in the SF than in the serum of animals with PGIA. These findings suggest that G-CSF, GM-CSF, and IL-6 are generated by joint-resident cells locally in both PGIA and RA, and presumably maintain the survival and suppressor capability of MDSCs in the SF.

The cells that we gave rise from mouse BM with the method detailed herein were real MDSCs, as they showed massive suppressive ability on both non-Ag-specific (polyclonal) and Ag-specific proliferation of T lymphocytes *in vitro*. Both MDSCs generated from BM precursors and those of SF origin suppressed the expansion of Ag-stimulated T lymphocytes, but BM-MDSCs had stronger potential than SF cells in inhibiting anti-CD3/CD28-induced polyclonal T lymphocyte expansion. That result led us to the conclusion that the suppressive capability of SF cells is selective, while BM-MDSCs have the ability to suppress T lymphocyte responses to Ag-specific and non-specific stimuli as well. Concerning BM-MDSCs, our data are in line with a previously reported study [81] in which "immature" myeloid cells with $CD11b^{+}Gr\text{-}1^{+}$ fenotype, originated from normal murine BM, inhibited the Ag-independent, anti-CD3/CD28- or mitogen-induced proliferation of T lymphocytes.

Although monocytic $Ly6G^{-}Ly6C^{hi}$ MDSCs have been implicated with having the ability to suppress T cell activity more strongly than the granulocytic subset [79], in contrary, when we depleted the $Ly6C^{hi}$ subpopulation, the BM-MDSCs retained their capacity to inhibit T cells functions. This is in line with our former [65] and other authors' [48, 82] findings that $Ly6G^{+}$ MDSCs with granulocytic phenotype is a subpopulation of cells with strong suppressor ability.

Our investigations to clarify the molecular mechanisms of BM-MDSC-mediated activity uncovered that inhibitors of iNOS were capable of reversing the Ag-specific and non-specific suppression of T lymphocyte proliferation as well. Consistently with this finding, iNOS was upregulated in BM-MDSCs at both mRNA and at protein levels. Moreover, in the supernatants of BM-MDCS cultures NO was detectable in high quantities. NO has the ability to inhibit T lymphocyte functions through chemical alteration of the TCR, amongst multiple other mechanisms, including inhibition of transcription factors and kinases implicated in the IL-2 receptor signaling pathway [83]. MDSCs from granulocytic subset have been found to exert inhibition of T lymphocytes via an arginase 1-dependent [48] or ROS-dependent [79] pathways. Nonetheless, granulocytic MDSCs present in the SF of inflammed murine joints [65] or in the BM [81] along with the MDSCs generated from mice BM *ex vivo* (present study) have definitely the ability to suppress T cell responses in a NO-dependent pathway. With regard to RA, in the sera and SF increased levels of NO were detected where the levels of NO were higher in SF than in the serum, suggesting local NO production within the joint [84]. The primary source of NO in the SF in RA patients could be cells with granulocytic phenotype that constitute the major cell population in SF [28], hence functioning as a resident granulocytic MDSCs.

Albeit precised characterization of the T lymphocyte signaling mechanism influenced by BM-MDSCs was not within the scope of this study, induction of T_{REG} cells as well as intracellular levels of IL-10 and IFNγ in $CD4^{+}$ T_H cells were measured. The levels of the T_H1 derived pro-inflammatory cytokine IFNγ was reduced, but interestingly so was the IL-10 level as well, an anti-inflammatory cytokine produced by T_H2 subsets. In murine models of cancer diseases induction of T_{REG} cell differentiation mediated by MDSCs has been published *in vitro* [85], but the proportion of T_{REG} cells was actually decreased, according to our data, in the presence of BM-MDSCs in PGIA.

One possibility of the immunosuppressive effect of the MDSCs is via induction of T_{REG} cells. This mechanism could be IFNγ and IL-10 dependent, but NO independent as reported by Huang B. et al [85]. However, in our animal model (PGIA) we could not detect

neither elevated IFNγ nor IL-10 levels. The proportion of T_{REG} cells was moderately but statistically decreased, however we could not test the potential of T_{REG} cells in this system. In CIA, another animal model of RA, the depletion of the $CD4^+CD25^+$ cells increased the severity of arthritis [86, 87]. In contrast, in PGIA, immunisation with PG did not increase the number of T_{REG} cells compared with naïve mice, moreover the adoptive cell transfer with T_{REG} cells did not prevent the symptoms of arthritis [88]. These results suggest that in PGIA T_{REG} cells does not represent the main immunoregulatory factor, hence the moderate decrease in their number caused by BM-MDSCs does not influence negatively the balance of pro- and anti-inflammatory factors.

When we tested the intracellular levels of IL-10, a T_H2-type anti-inflammatory cytokine, within the $CD4^+$ T cells, to our surprise, we also found decreased levels expressed by BM-MDSCs. This result differs from a recently published study by Park et al [89]. In that study, the suppressive ability of MDCSs originated from spleens of arthritic mice with CIA was IL-10 dependent *in vitro,* as well as *in vivo*. On one hand, these authors tested mRNA expression of different anti-inflammatory molecules in the MDSCs and the results were compared with mRNA levels within monocytes. It was found that mRNA levels of IL-10 was higher in MDSCs. On the other hand, when an IL-10 inhibitor was added to the co-cultures of T cells and MDSCs, induction of T_{REG} cells by MDSCs was antagonized by the anti-IL-10 agent. Moreover, upon adoptive cell transfer with MDSCs isolated from IL-10 deficient mice failed to prevent the development of CIA. These results underline that production of IL-10 by MDSCs from arthritic mice with CIA might play an important role in the induction of T_{REG}s [89]. The differences between the two studies may be explained by the difference of the two animal models applied, PGIA and CIA. They used MDSCs isolated from spleens by sorting cells based on cell surface markers, we used MDSCs generated *in vitro*. We only measured IL-10 levels within $CD4^+$ cells not in MDSCs, a possibility that could have further clarify the molecular mechanism of immunoregulatory effect of BM-MDSCs. Yet, we cannot rule out the possibility that BM-MDSCs also express high levels of mRNA of IL-10 [89].

The fact that all three $CD4^+$ T cell subpopulations (T_H1, T_H2, $CD4^+CD25^+FoxP3^+$) decreased in the presence of BM-MDSCs suggests that the suppressive effect of BM-MDSCs may extend to several T lymphocyte subpopulation, thus is not selective.

There are a number of publications that described successful treatment with *in vivo* transfer of MDSCs in different pathological conditions. Highfill et al [59] reported generation of MDSCs *in vitro* from the BM of cancer-free murins by adding G-CSF, GM-CSF, and IL-13. These MDSCs suppressed responses to allogeneic cells *in vitro* and *in vivo* as well (in

graft-versus-host disease) [59]. In another study transfer of sorted $CD11b^{+}Gr\text{-}1^{+}$ cells, in an animal model of inflammatory bowel disease, abolished the signs of enterocolitis, suggesting a direct immunoregulatory mechanism through NO pathway [90]. In type I diabetes it was described that MDSCs considerably delayed or even suppressed the disease onset by inhibiting autoreactive T lymphocytes and increasing the differentiation of T_{REG}s [91]. In a murine model of multiplex sclerosis, cell transfer of granulocytic MDSCs from spleen slowed down the onset of the disease and decreased the severity via inhibition of encephalitogenic T_H1 and T_H17 lymphocytes [82]. Fujii et al [48] published that in CIA MDSCs (mostly with granulocytic characteristics) accumulate in the spleens of animals at the peak of the arthritis. These data are in line with our previous findings that MDSCs accumulate in autoimmune arthritis, however, in our study in PGIA, suppressive MDSCs were detected in the SF, not in the spleen [65]. In CIA spleen-derived MDSCs with granulocytic phenotye suppressed anti-CD3/CD28-induced T lymphocyte proliferation, but the Ag (type II collagen)-specific immune responses were not evaluated [48]. In the animal model of autoimmune arthritis used in our study MDSCs from SF showed suppression on T cells in an Ag-specific way and they were lack of the ability to influence the Ag-independent system, whereas $CD11b^{+}$ myeloid cells derived from the spleens at the peak of inflammation did not exerted suppressive ability in either of these *in vitro* settings [65]. On the contrary, in both Ag-specific and non-specific manner, as reported in the present study, MDSCs generated from the BM of naïve mice had suppressive capacity. The functional heterogeneity of MDSCs may unfold this obvious discrepancy [92]. There is a possibility that overlapping and distinct manners of suppressive effects occur, depending not only on the specific cytokine milieu but also the experimental model studied. Both of these factors may support and fine-tune the MDSCs.

We performed adoptive cell transfer in PGIA after the first signs of arthritis with MDSCs. After a single injection of such cells into SCID mice prevented further joint damage and suppressed progression of the disease. In order to ascertain if BM-MDSCs exerted immunomodulatory effects *in vivo*, serum Abs and Ag-specific T cell proliferation were assessed in the recipient mice. Data proved that both B- and T lymphocyte responses were significantly suppressed in the treated group of SCID mice.

As we discussed earlier, T cells play a central role in the initiation and the perpetuation of RA, but B lymphocytes are also important players in the pathomechanism by producing autoantibodies (e.g. RF, ACPAs and others). The immunoregulatory capacity of MDSCs is primarily via targeting T cell functions, but B cells are also affected in this process. After adoptive cell transfer of BM-MDSCs in the SCID mice, we also evaluated different

autoantibody levels, and we found a significant decrease in the anti-PG-IgG1 levels, hence we proved that MDSCs generated from BM *in vitro* were actually able to inhibit B cell functions as well. Not much data can be found about the relationship of MDSCs and B lymphocytes in autoimmune arthritis. However, Crook et al [93] investigated B cell functions in the presence of MDSCs in CIA. According to their results, upon co-culturing M-MDSCs isolated from BM of arthritic mice with B cells, MDSCs were able to inhibit B cell proliferation as well. This effect was NO, PGE_2 and cell-cell contact dependent. Moreover, adoptive transfer of M-MDSCs prevented joint inflammation in wild type mice and lower levels of autoantibodies were detected as well [93].

Experiments to elucidate the tracking of transferred BM-MDSCs *in vivo* uncovered that these cells predominantly accumulated in the SF and BM. These environments consists locally produced cytokines and myelopoietic growth factors that support best the survival of MDSCs. Moreover BM-MDSCs were present in the blood samples 19 days after their transfer that also suggested active trafficking of MDSCs between the SF and BM. This finding indicated that BM-MDSCs suppressed progression of inflammation by limiting the expansion of pathogenic T lymphocytes in the peripheral joints, in the BM and, to a lesser degree, in the secondary lymphoid organs of SCID mice.

Tracking of BM-MDSCs in the SCID mice after i.p. injection of these cells proved that MDSCs actually do accumulate at the site of the inflammation, within the SF in this case. This finding underline the importance of the migration of MDCSs into the joint. This conclusion is in line with the result of Crook et al [93]. They investigated an aggravated form of CIA in $CCR2^{-/-}$ mice. CCR2 is a key chemokine receptor in the migration of monocytes/macrophages, and the absence of such molecule results an exacerbated form of CIA. Adoptive transfer of M-MDSCs into the $CCR2^{-/-}$ mice resulted the amelioration of the disease suggesting that M-MDSCs play a key role in the regulation of inflammation within the joint. Although BM-MDSCs are dominated by a granulocytic subtype of MDSCs they also contain monocytic precursors as well [93].

To our best knowledge, our group was one of the firsts who performed adoptive cell transfer with MDSCs in an animal model of RA. Nonetheless, there are more and more data proving the therapeutic effect of MDSCs in autoimmune arthritis. As mentioned before, Fujii et al [48] transferred MDSCs isolated from spleen of mice with arthritis (mainly of granulocytic subtype) and it showed a beneficial effect by preventing development of CIA, and depletion of such cells aggravated the diseases. The underlying mechanism was independent of induction of T_{REG} cells [48]. Also in the CIA model, Crook et al [93]

transferred BM-derived M-MDSCs and also managed to prevent joint inflammation in wild type and in $CCR2^{-/-}$ mice. T cell functions were inhibited via NO and IFNγ dependent, but IL-17 independent mechanisms. B cell functions were also assessed and the suppression of autoantibody production was actually PGE_2 dependent. Notably, in this experiment $Ly6G^+$ cells were ineffective [93]. In a recently published study by Park et al [89], the suppressive capacity of different MDSC subtypes (M-MDSCs and G-MDSCs) were evaluated, and all MDSCs were effective in decreasing arthritis severity scores in CIA regardless of the subtype. Besides up-regulation of iNOS and arginase-1 gene in MDSCs, IL-10 was also overexpressed, and IL-10 dependent induction of T_{REG} cells was determined [89]. Interestingly, some authors described pro-inflammatory effects of MDSCs. Guo et al [94] described a positive correlation between MDSC expansion in mice with CIA. Moreover, cell transfer with $CD11b^+Gr1^+$ cells sorted from spleen of mice with CIA in fact facilitated joint inflammation while depletion of such cells ameliorated arthritis. This group also detected overexpression of TNF-α and IL-1β in MDSCs proving their inflammatory activation. The mechanism of the pro-inflammatory effect was via induction of T_H17 cells [94]. The difference between the aforementioned contradictory results can be best explained by the plasticity and multifaceted nature of MDSCs. The results are influenced by the animal model applied, the tissue or anatomical site of their isolation, the precise cytokine microenvironment and the actual disease activity [95]. Based on the previous findings we emphasize the importance of the source of MDSCs transferred. A controlled, reproducible method for generating anti-inflammatory MDSCs seems to be paramount for any further investigation targeting the exploitation of therapeutic potential of such cells.

In mice with PGIA the transplantation of syngeneic BM was able to restore immune homeostasis thus suppressed arthritis progression in an earlier published study [96]. In this experiment accumulation of T_{REG} cells was found after BM transfer in the recipient animals. The origin of the T_{REG} cells was not clarified if it was of donors or of recipients, however, the indicated mechanism of disease suppression was T_{REG} differentiation mediated by BM [96]. While the BM may play role as a reservoir of T_{REG} lymphocytes [97] it also contains notable numbers of MDSCs and their precursors [81, 97]. It cannot be ruled out that MDSCs present in the BM took part in the suppression of autoimmune mechanisms and thus reduced the severity of the disease upon BM transplantation into mice with PGIA.

A study by Jiao et al [51] described in the peripheral blood of patients with RA expansion of MDSC-like cells when compared with the samples from healthy controls, altough the effects of the MDSCs were not evaluated [51]. It is a possibility that MDSCs of

SF origin limit the expansion of joint-homing (pathogenic) T lymphocyte in both RA and animal models of the disease. Additionally, only very low proportions of T lymphocytes has been described in the SF of the inflammed joints of both RA patients and mice [24, 25, 98]. Furthermore, it was reported that T lymphocytes isolated from the SF of RA patients showed hypo-responsiveness to mitogenic stimuli as compared to peripheral blood T lymphocytes of the same individual [72]. It is likely, therefore, that SF-MDSCs suppress locally the expansion of T lymphocytes, hence assist to the resolution of the arthritis. Moreover, in mice with CIA it was described that *in vivo* applying the anti-Gr-1 mAb RB6-8C5 to deplete MDSCs caused a delay in the resolution of inflammation in the joints [48]. Nonetheless, when MDSCs enter the inflammed joints at the early stage of the disease may also provoke collateral tissue damage via NO-release or other noxious products, thus acting as a "double-edged sword" [99]. Assesment of the characteristics and effects of MDSCs present in RA patients in peripheral blood, SF, BM, secondary lymphoid organs or other anatomical sites could significantly contribute to the understanding of the role of MDSCs in the regulation of autoimmunity and joint pathology in RA.

In summary, in this study we report an *in vitro* protocol for generating MDSCs from mouse BM in large quantities in a reproducible and controlled method. We described that BM-MDSCs in mice, in part, sharing common features with MDSCs present in the SF of mice with PGIA significantly limit T lymphocyte responses *in vitro* and *in vivo*. These data contribute to a better understanding of an innate control mechanism that plays part in the regulation of immune responses and arthritis severity in an animal model of RA and most likely also in human patients. Albeit further studies are warranted, our findings also indicate that *in vitro* enrichment of the BM in MDSCs could increase the therapeutic potential of autologous BM transplantation in patients with severe, treatment-resistant RA.

6.2 Identification of MDSCs in the synovial fluid of RA patients

In contrast to several studies carried out in animal models described above, there have been much fewer reports on the role of MDSC in human RA. It was reported by Jiao et al [51] that the frequency of MDSC-like cells in the RA patients' peripheral blood was increased but not in the samples from healthy individuals. A negative correlation was found between the numbers of T_H17 cells and MDSC-like cells. Unfortunately, MDSC-like cells were

characterized only by expression of phenotypic markers, and the biological activity of these cells toward T lymphocytes was not evaluated in that report [51].

In contrast, in a recently published report investigating MDSC expansion in RA by Guo et al [94], MDSCs were described as a potentially pro-inflammatory factors in the pathomechanism of RA. These authors found elevated numbers of circulating MDSCs in patients with high disease activity but not in patients with low disease activity and healthy controls. After the publication of our human data regarding RA and MDSCs they also detected MDSCs in SF of RA patients. A positive correlation was described between the number of joint resident MDSCs and IL-17 levels suggesting that MDSCs actually contribute to promote T_H17 differentiation thus contributing to the development of inflammation within the joint [94].

According to our understanding in this study we describe for the first time that MDSC-like cells can be detected in the SF of RA patients as well. As these cells have the ability to inhibit *ex vivo* induced proliferation of autologous T cells confirms that they are real MDSCs.

The dominating phenotype amongst MDSC-like RA SF cells was $CD11b^{+}CD33^{+}HLA\text{-}DR^{lo/-}CD14^{-}CD15^{+}$ (granulocytic) subset with morphology of neutrophils. However, in a small proportion we also found a $CD11b^{+}CD33^{+}HLA\text{-}DR^{lo/-}CD14^{+}CD15^{-}$ (monocytic) subpopulation in the SF samples of RA patients. The association between phenotype and function of MDSCs is still a subject of an ongoing debate. As we and others [52, 92] suggested both monocytic and granulocytic MDSCs have been found to exhibit different immune suppression mechanisms dependig on the tissue and disease. Furthermore, in humans among both mature neutrophil subset (with segmented or even hyper-segmented nuclei) and "immature" neutrophil cells (with band-shaped nuclei) have been determined a subset with granulocyte phenotype and suppressive ability [76]. As we reported earlier, in mice with PGIA the vast majority of SF cells with origin of arthritic joints were also granulocytic cells with neutrophil morphology. The suppressive capacity of SF cells was retained after negative selection of the minor monocytic MDSC subset [65]. In another animal model of RA (in CIA) granulocytic MDSCs were isolated from the spleens of arthritic mice [48]. It was reported that these cells inhibited the proliferation of T lymphocytes *in vitro*, and decreased the severity of the arthritis upon cell transfer *in vivo* [48].

Altogether, these findings and our results detailed in this study indicate that cells with immune suppressive capacities with the morphologic and phenotypic features of neutrophils are present in the SF of RA patients.

Although Guo et al [94] described a positive correlation between the numbers of MDSCs in RA patients, disease activity and T_H17 cells concluding that MDSCs might have a pro-inflammatory role, it is still possible that the expansion of such cells is a consequence of the inflammation rather than a causative factor. Unfortunately, in our present study, we could not assess the correlation between the actual DAS28 values and the numbers of MDSCs. However, based on the fact that the majority of the samples investigated came from patients with active disease our results reflect the role of MDSCs in an active state of the disease, and at least *in vitro*, they clearly expressed suppressive capacity towards T cells suggesting an anti-inflammatory role.

Alike SF cells originated from mice with PGIA [65], in present study we described that SF cells from RA patients were much more effective in suppressing Ag-specific proliferation of autologous T lymphocytes compared with the non-Ag-dependent (with anti-CD3/CD28 Ab) setting. Nonetheless, SF MDSCs from patients with RA, unlike SF cells from mice, had the ability to inhibit significantly on the robust proliferation of anti-CD3/CD28-stimulated T cells. These results suggest that SF MDSCs are a non-selective suppressors of T lymphocyte expansion, and also raise the question whether the difference in suppressive potency described in the Ag-specific versus non-specific stimuli may only be due to the difference in the response's magnitude of T cells in these two systems.

The possible effector pathways of MDSC-mediated suppression are the synthesis of nitric oxide (NO) by inducible NO synthase, L-arginine depletion via arginase-1, and various oxygen radicals production [47]. All of these mechanisms may exert negative influence on the cell cycle and CD3-related signaling in T cells [76]. We revealed that NO production is involved in the primary pathway of immune suppression by granulocytic SF cells in mice [65]. Since the patient samples available for these experiments were unfortunately limited, the investigations to uncover the precise suppressive mechanisms employed by RA SF cells was unfortunately also limited. Notably, increased levels of nitrite (formed from NO) have been detected in the SF of RA patients [84] supporting the likelihood that production of NO may be the underlying mechanism of suppressing T cell proliferation in this case.

Supporting factors of myelopoiesis like GM-CSF, G-CSF, and IL-6 have been involved in the induction and expansion of MDSCs [47, 53, 77]. Indeed, these cytokine and growth factors can be detected at elevated levels in the SF of RA patients [78] thus providing a supportive environment in which MDSCs can thrive. Additionally, the broadly described "hypo-responsiveness" of T lymphocytes in the SF of RA patients to mitogenic stimuli (as compared to the normal responsiveness of blood T lymphocytes from peripheral blood sample

of the same RA patient) [72] may partially be due to the longstanding exposure of T cells to MDSCs within the inflammed joint. Despite that T_{REG} cells with $CD4^+CD25^+FoxP3^+$ phenotype may be present in the joint exudate in RA [24] the inflammatory milieu considerably reduces the ability of these cells to suppress the activation and expansion of effector T lymphocytes in the SF or *ex vivo* [24, 25].

In the CIA model of RA intravenously injected (systemic cell transfer) MDSCs of spleen origin resulted reduced numbers of $CD4^+$ T cells and better arthritis severity scores of the recipient mice [48]. Moreover, MDSC-depletion *in vivo* restrained the spontaneous resolution of the arthritis [48]. Similarly, our study show that cell transfer of BM-MDSCs alleviated the joint inflammation in mice with PGIA and suppressed antigen-specific T cell responses and serum antibody levels. We suggest that in the joint exudate of RA patients MDSCs are present and while they might provoke collateral damage to joint tissues, they also act as negative regulators of T cell expansion locally in an attempt to break the vicious cycle of autoimmunity and inflammation.

7 CONCLUSIONS

At the beginning of our studies, we reviewed the molecular pathogenesis of arthritis. Genetic factors, autoimmunity and environmental factors trigger the onset of synovial inflammation. A high amount of data has been collected with respect to the role of HLA and non-HLA genes in susceptibility to RA. Our knowledge of the pathogenesis of RA lead to the development of various targeting strategies. However, not all aspects of arthritis pathogenesis have become delineated. Therefore, we turned our attention to the recently described MDCSs, their role in disease pathogenesis and prospects of therapeutic use.

In this work we report an *in vitro* protocol that is suitable to generate large numbers of MDSCs from mice BM in a controlled and reproducible mode. We describe that mouse BM-MDSCs, in part correspond to MDSCs present in the joint exudate of mice with PGIA, and have the ability suppress T cell responses *in vitro* and *in vivo*. Our data contribute to a better understanding of an innate control mechanism that affects the regulation of immune responses and arthritis severity in a murine model of RA and presumably also in human RA patients. Although further studies are warranted, our results also suggest that therapeutic outcome of autologous BM transplantation in patients with treatment-resistant, severe RA could be improved by *in vitro* enrichment of the BM in MDSCs. We also showed for the first time that MDSCs are present in the SF of RA patients. The dominant population of RA SF MDSCs similarly to those we detected in the mouse inflammed joint exudate in PGIA, exhibits neutrophil morphology and phenotype. The effect of MDSCs in this case seem to be non-selective as these cells significantly inhibit both the anti-CD3/CD28 Ab-induced and alloAg-induced proliferation of autologous blood T cells. We propose that MDSCs in the RA SF have the potential to restrict the expansion of joint-infiltrating (and most likely pathogenic) T cells. This study represents the first step of investigations into the role of MDSCs (present in the inflamed joints and perhaps at other sites) in controlling autoimmune T cell responses in RA.

8 New findings and clinical relevance

1. In this Ph.D. project we developed an *in vitro* method to generate MDSCs in PGIA, an animal model of RA. From bone marrow cells of naive mice using GM-CSF, G-CSF, and IL-6 we could obtain cells that were similar to MDSCs found previously in the SF of mice with PGIA. The dominant phenotype was $Ly6C^{int}Ly6G^{high}$, mainly granulocytic MDSCs, and *in vitro* BM-MDSCs suppressed very potently Ag-dependent and Ag-independent T cell proliferation in a NO mediated manner.
2. These BM-derived MDSCs showed therapeutic efficacy upon injection into SCID mice developing adoptively transferred PGIA. BM-MDSCs prevented the clinical signs of joint inflammation and also T and B cell responses. The resolution of histopathological signs of inflammation were also proved in the joint of treated SCID mice. Tracking these cells we described that MDSCs do accumulate at the site of inflammation (and also in bone marrow).
3. We also identified cells with MDSC-like phenotype and immunosuppressive activity in the SF of RA patients. We demonstrated the dominance of $CD11b^{+}CD33^{lo/hi}HLA\text{-}DR^{lo/-}CD14^{-}CD15^{+}$ (granulocytic) MDSC-like cells in RA SF. SF cells from the RA patients potently inhibited Ag-specific and anti-CD3/CD28-induced proliferation of autologous T cells.

Our results suggest that MDSCs could represent a novel therapeutic tool in RA. Our *in vitro* method to generate MDSCs provide an excellent basis to further examine the therapeutic potential of such cells in autoimmune arthritis. Our data regarding human RA may contribute to a better understanding of a detail less studied of the pathogenesis of RA.

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10 SUMMARY

The “Bermuda triangle” of genetics, environment and autoimmunity is involved in the pathogenesis of rheumatoid arthritis (RA). Autoimmunity is thought to arise as a result of dysregulation of adaptive immune responses, but defective function of the innate immune system may also contribute to the loss of control in adaptive immunity.

Myeloid-derived suppressor cells (MDSCs) constitute a cell subset that is part of innate immune system with the ability to suppress T lymphocyte responses. We previously described that MDSCs with a granulocytic phenotype are present in the synovial fluid (SF) of mice with proteoglycan (PG)-induced arthritis (PGIA), a T lymphocyte-dependent murine model of RA. However, the small number of MDSCs in the joint exudate that could be isolated from mice precluded further experiments to study the potential therapeutic effect of such cells. The aim of our animal studies was to develop an *in vitro* method for generating MDSCs (with the resemblance to MDSCs present in SF) that enables us to test the therapeutic potential of such cells in PGIA. Altough most data regarding MDSCs come from studies with cancer patients and tumor-bearing animals, recent data suggest that MDSCs also accumulate in autoimmune diseases and their animal models as well.

Murine bone marrow (BM) cells were cultured for 3 days with a cytokine mixture constituted of granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6). Microscopy, flow cytometry and biochemical assays were used to analyse and characterize these cells. To test the suppressor capacity of these BM-MDSCs T cells proliferation assays were carried out. To study the potential therapeutic effect of BM-MDSCs, SCID mice at the early stage of adoptively transferred PGIA were injected with BM-MDSCs. Changes on the clinical course and also PG-specific immune responses were analized compared to the non-treated group.

In the human studies synovial exudate form RA patients was examined using flow cytometry with antibodies of surface markers of MDSCs and the cell morphology was analized with microscopic techniques. The suppressor capacity of these cells was also evaluated *ex vivo* by T cell proliferation assays (using autologous peripheral blood T lymphocytes). To test the ability of RA SF cells on the proliferation of autologous T cells we carried out both antigen-specific (allogeneic cells) and antigen-nonspecific (anti-CD3/CD28 antibodies) induction systems. BM cells from mice became enriched in MDSC-like cells cultured in the presence of GM-CSF, G-CSF and IL-6, although compared with MDSCs found in SF demonstrated greater phenotypic heterogeneity. BM-MDSCs significantly

suppressed both antigen-specific and polyclonal T cell proliferation primarily through nitric oxide release. Cell transfer with BM-MDSCs into SCID mice with PGIA alleviated the severity of the arthritis, inhibited Ag-specific T cell responses and decreased serum anti-PG levels. In the SF from RA patients MDSC-like cells could be detected with a predominantly granulocyte (neutrophil)-like phenotype and morphology. Upon co-culture RA SF cells with anti-CD3/CD28-stimulated autologous T cells, significant inhibition was detected. The suppressing capacity of RA SF cells was more profound, compared side by side, on the alloantigen-induced than the anti-CD3/CD28-induced proliferation of autologous T lymphocytes.

Our *in vitro* enrichment method result an SF-like, but controlled milieu for generating BM myeloid precursors into MDSCs that profoundly inhibit both T cell responses and the progression of arthritis in a murine model of RA. Our data suggest that MDSCs enriched in BM might contribute to enhance the therapeutic efficacy of BM transplantation in RA.

We have proved that granulocytic (neutrophil-like) MDSCs are present in the SF of RA patients. Although these cells might be commonly regarded as inflammatory neutrophils, our studies suggest that the presence of neutrophil-like MDSCs in the SF is likely beneficial, as these cells have the ability to limit the expansion of joint-infiltrating T cells in RA.

11 ÖSSZEFOGLALÁS (Summary in Hungarian)

A genetika, a környezeti faktorok és az autoimmunitás "Bermuda háromszöge" fontos szerepet játszik a reumatoid arthritis (RA) patogenezisében. Az autoimmunitás az adaptív immunválasz diszregulációjának eredményeképpen jön létre, de a természetes immunrendszer hibás működése is közrejátszhat az adaptív immunrendszer szabályozásának kisiklásában.

A myeloid eredetű szuppresszor sejtek (angol irodalomban: myeloid derived suppressor cells, MDSCs) a veleszületett immunrendszer részét képezik és a T-sejt funkció szuppressziójára képesek. Egy korábbi munkánkban bizonyítottuk, hogy granulocyta fenotípusú MDSC-k vannak jelen a proteoglikán-indukált arthritis-es (PGIA) egerek ízületi folyadékában, amely az RA ismert T-sejt dependens autoimmun állatmodellje. Ezen sejtek terápiás lehetőségeire irányuló vizsgálatoknak gátat szab az, hogy a kísérleteinkben használt állatmodell ízületi folyadékából nyerhető MDSC-k száma limitált.

Jelen munkánk célkitűzése az volt, hogy kidolgozzunk egy *in vitro* módszert, amely segítségével hasonló fenotípusú MDSC-ket nyerjünk, mint amilyen fenotípusú sejtet karakterizáltunk PGIA-ben, valamint hogy ezen sejtek terápiás potenciálját vizsgáljuk. Az utóbbi évek kutatásai alapján a MDSC-k nemcsak a tumoros betegek szervezetében halmozódnak fel, hanem az autoimmun kórképekben szenvedő betegek szervezetében, és ezek állatmodelljeiben is.

Kísérleteink során az egerekből izolált csontvelősejteket 3 napig tenyésztettük granulocyta-makrofág kolóniastimuláló faktor (GM-CSF), interleukin-6 (IL-6), valamint granulocyta-kolóniastimuláló faktor (G-CSF) jelenlétében. Az ilyen módon nyert sejtek fenotípusát áramlási citometriával, mikroszkópos módszerekkel, és biokémiai módszerekkel vizsgáltuk, az immunszuppresszív képesség tanulmányozása pedig T-sejt szuppressziós assay-vel történt. SCID egerekben a PGIA klinikai tüneteinek csökkentésére az *in vivo* immunszuppresszív képesség teszteléséhez adoptív sejttranszfert hajtottunk végre, melynek változását az arthritis súlyossági fokában, valamint a PG-specifikus immunválasz monitorozásával mértük le. Ezen kívül RA-es betegek ízületi folyadékában található sejtek morfológiáját is vizsgáltuk áramlási citometriával, olyan sejtfelszíni markermintázatokat keresve, amelyek a MDSC-kre jellemzőek. Vizsgáltuk továbbá ezen sejtek autológ perifériás T-sejtekkel szembeni szuppresszív hatását *ex vivo* kísérletekben, ahol antigén-specifikus (allogén sejtekkel) és nem antigén-specifikus (anti-CD3/CD28 antitestekkel) módon történő T-sejt proliferáció gátlást határoztunk meg.

A GM-CSF, IL-6, G-CSF jelenlétében tenyésztett csontvelősejtekben felhalmozódott egy olyan sejtpopuláció, amely rendelkezett a potens MDSC-k tulajdonságaival, habár a fenotípus tekintetében nagyobb heterogenitást mutattak, mint a synoviális folyadékból izolált MDSC-k. Kimutatható volt ezen sejtek T-sejtekre irányuló szuppresszív hatása, mely antigén-dependens és antigén-independens módon is szignifikánsnak adódott. A szuppresszió molekuláris mechanizmusa mindkét esetben az indukálható nitrogén-monoxid szintáz fokozott expressziójával, illetve emelkedett nitrogén-monoxid szintekkel magyarázható. A MDSC-kkel történő transzfer során a kezelt SCID egerek csoportjában szignifikánsan csökkent az ízületi gyulladás, a PG-specifikus T-sejt válasz, valamint a szérum anti-PG antitest szintjei is.

Sikerült kimutatnunk, hogy a RA-es betegek ízületi folyadékából MDSC-k izolálhatók, melyekre jellemző, hogy döntően granulocytoid fenotípussal rendelkeznek, és szignifikánsan csökkentik a T-sejt proliferációt. Összehasonlítva ezen sejtek T-sejt proliferációs potenciálját antigén-dependens és antigén-independens módon, a szuppresszió mértéke sokkal nagyobb volt az alloantigén-indukált rendszerben.

Munkánk során kidolgoztunk egy olyan *in vitro* módszert, amely segítségével csontvelői sejtekből kontrollált körülmények között potens MDSC-k állíthatók elő. Ezen sejtekkel hatékonyan tudtuk szuppresszálni nemcsak a T-sejt válaszokat, hanem az ízületi gyulladás klinikai tüneteit is a RA egy ismert állatmodelljében. Eredményeink előrevetítenek egy újabb terápiás lehetőséget terápiarezisztens RA-s betegek számára, amely hasznosítható lehet a későbbiekben a csontvelő transzplantáció területén. Kimutattuk, hogy az MDSC-k jelen vannak a RA-s betegek ízületi folyadékában lévő gyulladásos neutrofil granulocyták között, valamint, hogy jelenlétük nagy valószínűséggel jótékony hatású, hiszen képesek limitálni az az autoreaktív T-sejtek expanzióját, ezáltal csökkenteni az ízületi gyulladás mértékét.

12 PUBLICATIONS (for thesis)

12.1 List of publications

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Candidate: Júlia Emese Kurkó
Neptun ID: HWVD71
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Kurkó, J. E.**, Vida, A., Glant, T. T., Scanzello, C. R., Katz, R. S., Nair, A., Szekanecz, Z., Mikecz, K.: Identification of myeloid-derived suppressor cells in the synovial fluid of patients with rheumatoid arthritis: a pilot study.
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List of other publications

4. Mikecz, K., Glant, T. T., Markovics, A., Rosenthal, K. S., **Kurkó, J. E.**, Carambula, R. E., Cress, S., Steiner, H. L., Zimmerman, D. H.: An epitope-specific DerG-PG70 LEAPS vaccine modulates T cell responses and suppresses arthritis progression in two related murine models of rheumatoid arthritis.
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11. Gaál, J., Varga, J., Szekanecz, Z., **Kurkó, J. E.**, Ficzere, A., Bodolay, E., Bender, T.: Balneotherapy in elderly patients: effect on pain from degenerative knee and spine conditions and on quality of life.
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Total IF of journals (all publications): 35,477
Total IF of journals (publications related to the dissertation): 9,679

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

15 May, 2018

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

12.2 Conference abstracts

1. **Kurkó, J.,** Szekanecz, Z., Szántó, S., Szűcs, G.: Ciprofloxacin-indukált szisztémás lupus erythematosus. Magyar Reumatológusok Egyesülete (MRE) 2006. évi vándorgyűlése, Debrecen. Magyar Reumatol. 47: 179, 2006.

2. **Kurkó J.,** Szekanecz Z., Ficzere A.: A balneoterápia és iszapkezelés immunológiai hatásai. Magyar Reumatológusok Egyesülete (MRE) 2007. évi vándorgyűlése, Szeged. Magyar Reumatol. 48: 141-142, 2007.

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4. Szántó S, Bodnár N, Szabó Z, Pethő Zs, Soós L, **Kurkó J,** Szűcs G, Szekanecz Z.: Spondylarthritis ankylopoeticás betegeink keresztmetszeti vizsgálata: a betegség aktivitásának, a betegek életminőségének, funkcionális állapotának és az alkalmazott kezelés hatásának összefüggése. Magyar Reumatológusok Egyesülete (MRE) 2008. évi vándorgyűlése, Budapest. Magyar Reumatol. 49:140-141, 2008.

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9. Mikecz K**, Kurkó J**, Ocskó T., Vida A., Tryniszewska B., Rauch A.T., Block J.A., Katz R.S., Nair A., Scanzello C.R., Glant T.T.: Suppression of Immune Responses and Joint Inflammation by Myeloid-derived Suppressor Cells in a T Cell-dependent Mouse Model of Rheumatoid Arthritis, American College of Rheumatology (ACR) 2012. évi vándorgyűlése , Washington D.C., USA

10. D.H. Ziemmerman, K. Mikecz, **J. Kurkó**, T. Glant, H. Steiner, R. Carambula, LEAPS peptide vaccination alters T cell phenotype and suppresses joint inflammation in murine model of rheumatoid arthritis, The American Association of Immunologists 2013. évi vándorgyűlése, Honolulu, Hawaii,USA

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12. **Kurkó J,** Mikecz K, Glant T, Szekanecz Z. Myeloid-eredetű szuppresszor-sejtek in vitro előállítása és terápiás lehetőségeinek vizsgálata rheumatoid arthritis egérmodelljében. Magyar Reumatológusok Egyesülete (MRE) 2013. évi vándorgyűlése, Debrecen. Magyar Reumatol. 54: 160, 2013.

13 KEYWORDS

13.1 Keywords

rheumatoid arthritis, proteoglycan-induced autoimmune arthritis, genetics, single nucleotide polymorphisms, HLA-DR, GWAS, myeloid-derived suppressor cells, T cells, synovial fluid, cell-based therapy

13.2 Tárgyszavak (Keywords in Hungarian)

rheumatoid arthritis, proteoglikán-indukált autoimmun arthritis, genetika, egypontos nukleotid-polimorfizmus, HLA-DR, GWAS, myeloid eredetű szuppresszor sejt, T sejtek, synoviális folyadék, sejtterápia

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