SHORT 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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The Examination takes place at the Library of Department of Ophtalmology,
Faculty of Medicine, University of Debrecen
on 19th November 2018 at 11:00 am

Head of the **Defense Committee**: Prof. András Berta, MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine,
Faculty of Medicine, University of Debrecen, on 19th November 2018 at 1:00 pm

1 INTRODUCTION

1.1 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. The etiopathogenesis of RA is initiated by the "Bermuda triangle" of genetic and environmental/lifestyle-related risk factors, as well as autoimmunity. In RA, the role of genetic factors have been approximated to 60%. Class II major histocompatibility antigens/human leukocyte antigens (MHC, HLA-DR), along with non-HLA genes have been involved in disease pathogenesis. We have recently reviewed the genetic background of RA. 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*. We have confirmed the association of *HLADRB1,* as well as *PTPN22* and *IL23R* SNPs in Hungarian RA patients. 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. Genetic and environmental factors, as well as autoantibody (ACPA)-mediated autoimmunity trigger synovitis, the central inflammatory event in RA, as well as joint destruction. Several inflammatory cells and mediators are involved in this process. T cells play a crucial role in the initiation and perpetuation of synovitis. 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. In addition, T cell recognition of citrullinated autoantigens (autoAgs) is also unevitable for the initiation of RA-associated autoimmunity. 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 may be the result of help to Ab-producing B lymphocytes provided by T helper (T_H) cells. 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. Neutrophils, that belong to the innate immune system, are major constituents in the RA synovial fluid (SF). 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, they may also impair joint T cells thus restricting their local expansion. Early diagnosis and prompt, effective medication are needed to prevent joint damage, functional disability and unfavorable disease outcome. 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. 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. 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. While transplantation of autologous bone marrow (BM) or hematopoietic stem cells have been of great promise in both refractory RA and therapy-resistant juvenile idiopathic arthritis, 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.

1.2 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, 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. In more detail, MDSCs were originally described in tumour-bearing mice and in humans with cancer more than two decades ago. 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. In RA and animal models of this disease, MDSCs interfere with synovial inflammation, T_H17 responses, as well as bone erosion. 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. 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. Therefore, we may regard the MDSC network as a universal tool developed by nature to control immune responses under various pathological conditions. 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. There are several protocols to generate MDSCs *in vitro* from murine BM precursors 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. 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. 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.

2 AIMS OF THE STUDY

In this Ph.D. thesis, various aspects of the role of myeloid-derived suppressor cells 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.

3 MATERIALS AND METHODS

3.1 Animal studies

3.1.1 Mice, immunization, and assessment of arthritis

Adult female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). 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 were used as a source of PG/G1-specific T cells. To induce arthritis, adult female wild type (wt) BALB/c mice were injected intraperitoneally (i.p.) with 100 μg of human PG protein 3 times 3 weeks apart. PG was extracted from human cartilage as described previously. A standard visual scoring system (0-16 per mouse) was used for the assessment of disease severity.

3.1.2 Collection of serum and cells/organs from mice

Blood for cell analysis and measurement of anti-PG Ab titers was drawn from mice. 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).

3.1.3 Generation of MDSCs and DCs from BM

MDSCs were generated from BM of naïve wt or EGFP-LysM-Tg BALB/c mice. BM cells were counted 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). 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).

3.1.4 Phenotypic analysis of mouse cells by flow cytometry

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). 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). 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).

3.1.5 Immunofluorescence imaging and cytospin preparations of mouse MDSCs

After BM-MDSCs were immunostained with fluorochrome-labeled mAbs to Ly6G and Ly6C, 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). 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).

3.1.6 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. Absorbance at 450 nm was read by a Synergy 2 ELISA reader (BioTek Instruments, Winooski, VT, USA).

3.1.7 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). Depletion of $Ly6C^{hi}$ (monocytic) cells from the total BM-MDSC population was carried out using an EasySep Biotin Selection Kit (StemCell Technologies). This resulted in the removal of essentially all $Ly6C^{hi}$ (but not $Ly6C^{int/lo}Ly6G^{hi}$) BM-MDSCs, as confirmed by flow cytometry.

3.1.8 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) 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. 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:

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

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

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). After amplification, samples were loaded onto 1.5% agarose gels.

3.1.10 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.

The membranes were stripped and re-probed with a HRP-conjugated mAb to β-actin at a 1:5,000 dilution to ensure equal sample loading.

3.1.11 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). Results were expressed as total nitrate concentration (μM).

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

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). When arthritis started to develop (day 15 after the first splenocyte transfer), mice were divided into two groups (n = 10 mice/group). 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. Mice were sacrificed on day 34 after the first cell transfer for determination of joint histopathology and PG-specific immune responses.

3.1.13 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.

3.2 Human studies

3.2.1 RA patients

Altogether 11 RA patients undergoing therapeutic joint fluid aspiration at two clinics. Informed consent was obtained from each of the participants. The 11 RA patients all donated SF, and 9 of them also donated blood. All patients had established RA according to the 2010 ACR/EULAR classification criteria 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).

3.2.2 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 then counted and used for phenotypic, morphologic, and functional analyses without further separation. An aliquot of SF cells was immunostained and processed for flow cytometry. 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).

3.2.3 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. 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). 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.

3.3 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.

4 RESULTS

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

In preliminary experiments, we sought to determine whether BM cells cultured for 3 to 7 days in the presence GM-CSF and IL-6 (also present in SF of RA patients) acquire an SF-MDSC-like phenotype. We also 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. 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). 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. The flow cytometry profile and morphology of BM-MDSC-like cells demonstrated greater heterogeneity than those of fresh SF cells, 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.

4.2 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%. Compared with SF cells BM-MDSCs were equally potent in suppressing T-cell proliferation. Depletion of the $Ly6C^{hi}$ monocytic subset from the BM-MDSCs 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). 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, whereas suppression by SF cells was very weak. 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.

4.3 The 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. However, BM-MDSCs lost much of their ability to suppress T-cell proliferation in both induction systems in the presence of the iNOS inhibitors, suggesting that the main MDSC product mediating T-cell suppression was NO.

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

BM-MDSCs demonstrated significant up-regulation of *Nos2* mRNA as compared with spleen cells, 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. The enzymatic activity of iNOS was assessed by measuring nitrite/nitrate concentration in supernatants of BM-MDSCs (cultured in the presence of DCs and rhG1 with or without T cells) and spleen cell cultures. Much higher levels of NO were detected in the supernatants of BM-MDSCs-containing cultures than in those of spleen cell cultures.

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

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 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, while, in sharp contrast, the scores of SCID mice transferred with BM-MDSCs remained low until the end (day 34) of the monitoring period. 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. Only mild synovial hyperplasia was observed in the ankle joints of SCID mice co-transferred with spleen cells and BM-MDSCs. PG-specific T-cell proliferation was significantly lower in the BM-MDSC-injected group. Serum levels of IgG1 isotype anti-PG Abs were also significantly reduced in the BM-MDSC recipient group. In a separate experiment, we assessed the distribution and subset composition of transferred $EGFP^+$ BM-MDSCs in various fluids and tissues of SCID mice with adoptively transferred PGIA 19 days after BM-MDSC injection (half of the optimal therapeutic dose). 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.

4.6 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. The predominant cell type in RA SF was $CD11b^+CD33^+HLA\text{-}DR^{lo/-}CD14^-CD15^+$ granulocytic MDSC-like cells, but a small population of the $CD11b^+CD33^+HLA\text{-}DR^{lo/-}CD14^+CD15^-$ monocytic subset was also present. MDSC-like cells constituted ~85% of all SF cells and ~95% of these MDSC-like cells belonged to the granulocytic subset.

4.7 RA SF cells significantly 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. Isotope incorporation (cpm) by unstimulated PBMCs or anti-

CD3/CD28-treated SF cells 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. We calculated the background-corrected proliferation ratios for the 9 patients tested. SF cell-mediated suppression of anti-CD3/CD28-induced T-cell proliferation was statistically significant ($p = 0.0039$, 95% CI = 0.4682-0.7495).

4.8 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 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. 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, and also significant ($p = 0.0087$) and more effective suppression of the Ag-induced (and more modest) proliferation of the same T cells. 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, 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.

5 DISCUSSION

5.1 *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. Data obtained from cancer patients and tumor-bearing animals provided the greatest amount of information on these cells. Lately, these cells have been identified in numerous autoimmune conditions characterized by excessive activation of adaptive immunity.

In our former paper, we demonstrated that in the PGIA model of human RA, SF contains a population of cells that resembles MDSCs. 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. This led us to seek a different opportunity to generate large amounts of MDSCs. 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.

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.

We have assumed in our former paper 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 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. Although monocytic $Ly6G^{-}Ly6C^{hi}$ MDSCs have been implicated with having the ability to suppress T cell activity more strongly than the granulocytic subset, in contrary, when we depleted the $Ly6C^{hi}$ subpopulation, the BM-MDSCs retained their capacity to inhibit T cells functions.

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. 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. 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, 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. This result differs from a recently published study by Park et al. 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. The differences between the two studies may be explained by the difference of the two animal models applied, PGIA and CIA.

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 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). 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. Fujii et al published that in CIA MDSCs (mostly with granulocytic characteristics) accumulate in the spleens of animals at the peak of the arthritis. 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. 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. 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.

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.

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.

There are more and more data proving the therapeutic effect of MDSCs in autoimmune arthritis. Interestingly, Guo et al 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. The difference between the contradictory results can be best explained by the plasticity and multifaceted nature of MDSCs.

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. 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. It is likely, therefore, that SF-MDSCs suppress locally the expansion of T lymphocytes, hence assist to the resolution of the arthritis.

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.

5.2 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 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.

In contrast, in a recently published report investigating MDSC expansion in RA by Guo et al, 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.

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.

Although Guo et al 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, 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.

Supporting factors of myelopoiesis like GM-CSF, G-CSF, and IL-6 have been involved in the induction and expansion of MDSCs. Indeed, these cytokine and growth factors can be detected at elevated levels in the SF of RA patients 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) 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 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*.

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.

6 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) are innate immune cells capable of suppressing T-cell responses. The goals of our animal studies were to develop an *in vitro* method for generating MDSCs similar to those found in SF and to reveal the therapeutic effect of such cells in PGIA. MDSCs have been shown to accumulate in cancer patients, but recent studies suggest that these cells are also present in humans and animals suffering from autoimmune diseases.

Murine bone marrow (BM) cells were cultured for 3 days in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-6 (IL-6), and granulocyte colony-stimulating factor (G-CSF). The phenotype of cultured cells was analyzed using flow cytometry, microscopy, and biochemical methods. The suppressor activity of BM-MDSCs was tested upon co-culture with activated T cells. To investigate the therapeutic potential of BM-MDSCs, the cells were injected into SCID mice at the early stage of adoptively transferred PGIA, and their effects on the clinical course of arthritis and PG-specific immune responses were determined.

SF cells from RA patients were studied by flow cytometry using antibodies to MDSC cell surface markers as well as by analysis of cell morphology. The suppressor activity of RA SF cells toward autologous peripheral blood T cells was determined *ex vivo*. We employed both antigen-nonspecific (anti-CD3/CD28 antibodies) and antigen-specific (allogeneic cells) induction systems to test the effects of RA SF cells on the proliferation of autologous T cells.

Murine BM cells cultured in the presence of GM-CSF, IL-6, and G-CSF became enriched in MDSC-like cells that showed greater phenotypic heterogeneity than MDSCs present in SF. BM-MDSCs profoundly inhibited both antigen-specific and polyclonal T-cell proliferation primarily via production of nitric oxide. Injection of BM-MDSCs into mice with PGIA ameliorated arthritis and reduced PG-specific T-cell responses and serum antibody levels. SF from RA patients contained MDSC-like cells, the majority of which showed granulocyte (neutrophil)-like phenotype and morphology. RA SF cells significantly suppressed the proliferation of anti-CD3/CD28-stimulated autologous T cells upon co-culture. When compared side by side, RA SF cells had a more profound inhibitory effect on the alloantigen-induced than the anti-CD3/CD28-induced proliferation of autologous T cells.

Our *in vitro* enrichment strategy provides a SF-like, but controlled microenvironment for converting BM myeloid precursors into MDSCs that potently suppress both T-cell responses and the progression of arthritis in a mouse model of RA. Our results also suggest that enrichment of BM in MDSCs could improve the therapeutic efficacy of BM transplantation in RA.

We have shown 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.

7 NEW FINDINGS, RESULTS

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.

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List of publications related to the dissertation

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