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Research Article Activation-dependent cell death of human monocytes is a novel mechanism of fine-tuning inflammation and autoimmunity

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In patients with juvenile idiopathic arthritis (JIA), increased release of IFN-y and GM-CSF in cells infiltrating synovial tissue can be a potent driver of monocyte activation. Given the fundamental role of monocyte activation in remodeling the early phases of inflammatory responses, here we analyze the GM-CSF/IFN- γ induced activity of human monocytes in such a situation in vitro and in vivo. Monocytes from healthy donors were isolated and stimulated with GM-CSF \pm IFN- γ . Monocyte activation and death were analyzed by flow cytometry, immunofluorescence microscopy, ELISA, and qPCR. T-cell GM-CSF/IFN-γ expression and monocyte function were determined in synovial fluid and peripheral blood from 15 patients with active JIA and 21 healthy controls. Simultaneous treatment with GM-CSF and IFN- γ induces cell death of monocytes. This cell death is partly cathepsin B-associated and has morphological characteristics of necrosis. Monocytes responding to costimulation with strong proinflammatory activities are consequently eliminated. Monocytes surviving this form of hyperactivation retain normal cytokine production. Cathepsin B activity is increased in monocytes isolated from synovial fluid from patients with active arthritis. Our data suggest GM-CSF/IFN-y induced cell death of monocytes as a novel mechanism to eliminate overactivated monocytes, thereby potentially balancing inflammation and autoimmunity in JIA.

Keywords: Arthritis · Autoimmunity · Caspase · Host defense ·Immune activation · Immune response

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Correspondence: Dr. Jan Däbritz e-mail: Jan.Daebritz@med.uni-rostock.de The pathogenesis of autoimmune joint diseases including juvenile idiopathic arthritis (JIA) involves a complex interplay of aberrantly activated innate and adaptive immune cells. In this context,

Introduction

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T cells are an important source of cytokines such as interferon gamma (IFN- γ). More recently, the importance of interleukin (IL)-23 and granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing T-helper (Th) 17 cells for autoimmunity has been established [1, 2]. GM-CSF production by T cells has been linked to several autoimmune inflammatory diseases, including multiple sclerosis (MS), myocarditis, and arthritis [3]. The precise mechanism by which GM-CSF promotes autoimmunity has not been described, but the existing literature identifies GM-CSF as a key cytokine by which Th17 cells might activate myeloid cells, thereby linking the adaptive with the innate immune system in autoimmunity [4]. These data were generated in mouse models and their relation to the human system is not fully established, but notably an enrichment of IFN-y/GM-CSF coproducing cells within the Th17 population in the inflamed joints of children with JIA has recently been demonstrated [5, 6].

Both IFN- γ and GM-CSF are potent inducers of monocyte activation. Sustained IFN-y signaling leads to classically activated proinflammatory (M1) macrophages and additionally has been shown to switch lineage committed anti-inflammatory (M2) macrophages back into a proinflammatory M1 phenotype [7]. Besides being a growth factor for granulocytes and macrophages, GM-CSF's major function is promoting the maturation and activation of monocytes and dendritic cells (DCs) as a proinflammatory cytokine [8]. Monocytes are cells with a high plasticity that respond to various stimuli with distinct activation and differentiation patterns [9]. Their activation is complex with iterative differentiation into different monocyte and macrophage subphenotypes resulting in partially opposing effector functions ranging from pro- to anti-inflammatory responses [10]. Tight control of these mechanisms is necessary to ensure that acute inflammatory reactions are self-limited and do not result in perpetuated autoimmunity. While over time the phenotype of monocytes may switch, it is conceivable that during acute inflammation monocytes undergo a process of priming. In this context, activated monocytes may receive early cytokine signals promoting their survival or cell death. To date, this aspect of immunoregulation has been almost exclusively described in the adaptive immune system. In peripheral T cells, a form of apoptosis induced by repeated T-cell receptor stimulation, known as activation-induced cell death (AICD), may be responsible for the peripheral deletion of autoreactive T cells [11]. AICD results from the interaction between Fas and Fas ligand, and activated T cells expressing both Fas and Fas ligand are killed either by autocrine or paracrine interactions [11, 12]. Thus, AICD is an important mechanism of regulating overwhelming activation to prevent autoinflammation and autoimmunity. However, a similar mechanism has not yet been described for monocytes.

We confirm the accumulation of IFN- γ /GM-CSF coproducing T cells in inflamed joints of children with JIA. In the light of pleiotropic functions and fundamental role of monocyte activation during early phases of inflammatory responses, we have analyzed the response of human monocytes to IFN- γ /GM-CSF in vitro. We demonstrate that this simultaneous coactivation promotes activation-dependent cell death of monocytes. The underlying cell

death pathway was identified as partly cathepsin B-dependent. Finally, we demonstrate an increased cathepsin B activity also in vivo in synovial monocytes from inflamed joints. Our data suggest GM-CSF/IFN- γ induced cell death of monocytes as a potential modulatory mechanism leading to counter-balancing inflammation and autoimmunity in JIA.

Results

Simultaneous stimulation of human monocytes with GM-CSF and IFN- γ induces cell death

Since costimulation of human monocytes with GM-CSF and IFN- γ leads to proinflammatory cell activation, we assessed the influence of GM-CSF on monocyte survival. We observed a relevant induction of cell death in a substantial proportion of GM-CSF/IFN-y cotreated monocytes (Fig. 1A). GM-CSF treatment had no effect on early stages of apoptosis assessed by annexin-V single positive staining (Fig. 1B, black bars). In addition to detailed kinetic experiments (Fig. 1B), we investigated the effects of different incubation times for GM-CSF and IFN- γ treatments. To this end, we treated monocytes with IFN- γ for 48 h with GM-CSF costimulation performed during the last 24 h. This approach showed no differences from the observed GM-CSF/IFN-y induced cell death of monocytes, as the proportion of annexin-V/PI double positive cells treated with GM-CSF and IFN-y was comparable to results obtained after 24 or 48 h of stimulation with both cytokines for the same duration (34.3 \pm 3.3 SEM versus 28.8 \pm 4.8 SEM; p = 0.439). Therefore, GM-CSF and IFN- γ costimulation robustly leads to cell death of a significant number of monocytes. Morphologic characteristics of GM-CSF \pm IFN- $\!\gamma$ stimulated and untreated monocytes for different time points are provided in Figure 2. Monocyte stimulation with GM-CSF + IFN- γ for 24 h induced morphologic changes such as cellular swelling and formation of balloon-like structures without signs of karyopyknosis and karyorrhexis. Cells containing hypodiploid nuclei were not present at any time point as assessed by Nicoletti assay indicating a necrotic form of cell death rather than late apoptosis (data not shown).

In addition, we validated the purity of isolated monocytes by flow cytometry using different cell surface markers specific for monocytes (CD14, CD16) and other immune cells including NK cells (CD56), T cells (CD3), granulocytes (CD66b), and B cells (CD19, CD20). We show that the purity of isolated monocytes is >90% and that the contamination with other immune cells is negligible (Supporting Information Fig. 1).

GM-CSF/IFN- γ costimulation induces cathepsin B-associated cell death

We next analyzed the functional contribution of caspase activation to GM-CSF/IFN- γ induced cell death by blocking caspasedependent pathways. Despite the inhibition of caspases and RIP1



Figure 1. Cell death assays and pathways. (A) Human monocytes from healthy controls were treated with GM-CSF + IFN- γ for 24 h or left untreated as a negative control (w/o). Cell death was measured by staining with annexin V (x-axis) and propidium iodide (PI, y-axis) as exemplarily shown in histoplots. (B) Human monocytes from healthy controls were treated with GM-CSF ± IFN- γ for 2, 4, 6, 12, 24, and 48 h or left untreated as a negative control (w/o). Cell death was measured by single staining with annexin V (black bars) and double staining with annexin-V and propidium iodide (white bars). (C) Human monocytes from healthy controls were treated with GM-CSF ± IFN- γ for 24 h or left untreated as a negative control (w/o). Cell swere precultured for 1 h with broad-spectrum caspase inhibitor z-VAD-fmk (50 µM), caspase-1 inhibitor z-yVAD-fmk (50 µM), cathepsin B inhibitor z-FA-fmk (50 µM), or RIP1 kinase inhibitor necrostatin-1 (Nec-1, 15 µM). Cell death was measured by double staining with annexin-V and propidium iodide. Gray bars represent double positive stained cells. (D) Human monocytes from healthy controls were treated for 24 h with either the medium control (w/o), GM-CSF ± IFN- γ , or 400 nM staurosporine (STS). For evaluation of caspase activity, caspase-specific FITC-labeled inhibitors were added and cells were stained with 7AAD and analyzed by flow cytometry. Additional control experiments are provided in Supporting Information Fig. 1B-D. Data are shown as mean + SEM (n = 3-9) and are pooled from six independent experiments. Significance (refers to control cells [w/o]): "p < 0.05; "*p < 0.01; "**p < 0.01."

kinase, GM-CSF/IFN- γ costimulation induced significant cell death of monocytes after 24 h compared with untreated or single treated monocytes (Fig. 1C). However, the GM-CSF/IFN- γ induced cell death was completely inhibited when we used a cathepsin B inhibitor (z-FA-fmk; Fig. 1C). We have performed additional experiments using a more selective cathepsin B inhibitor (CA074) and show that both cathepsin B inhibitors used (z-FA-fmk and CA074) are capable to block GM-CSF/IFN- γ induced cathepsin B activity (Supporting Information Fig. 2A). Finally, we determined caspase-3, caspase-8, and caspase-9 activity using fluorescencelabeled inhibitors in addition to annexin-V and 7AAD staining. We found that caspase activities were significantly reduced in early apoptotic cells (7AAD negative) treated with GM-CSF + IFN- γ whereas 7AAD-positive cells showed no significant differences in caspase activities compared with control cells (Fig. 1D). Therefore, the observed GM-CSF/IFN- γ induced cell death of monocytes is a caspase- and RIP1 kinase-independent but cathepsin B-mediated cell death pathway with the morphological characteristics of necrosis. Methods and results of additional control experiments are provided in Supporting Information Fig. 2. We show that the inhibitors we used to block specific cell death pathways were effective. Z-FA-fmk and the more selective cathepsin B inhibitor CA074 were capable to block not only nigericininduced cathepsin B activity (Supporting Information Fig. 2B) but also GM-CSF/IFN- γ induced cathepsin B activity (Supporting Information Fig. 2A). Moreover, also LPS/z-VAD-fmk induced



Figure 2. Microscopic analysis of cell death. Human monocytes from healthy controls were treated with GM-CSF \pm IFN- γ for 4, 8, 12, 24 h or left untreated as a negative control (w/o). Cell death was analyzed by staining with the phosphatidylserine sensor Apoxin Green (green) and propidium iodide (red). Nuclei were counterstained with Hoechst 33258 (blue). Images are shown at magnifications of 400× (inserts) and 200× with scale bars representing 20 and 50 μ m, respectively. Images are representative of three independent experiments.

RIPK1-dependent cell death could be blocked not only by the RIPK1-specific inhibitor necrostatin-1 as shown by directly measuring cell death (Supporting Information Fig. 2C) but also by measuring cell viability (Supporting Information Fig. 2D). Caspase 1 activity was induced by LPS/nigericin cotreatment of monocytes and the caspase 1 inhibitor z-yVAD-fmk was capable to reduce caspase 1 activity as measured by IL-1ß release from monocytes (Supporting Information Fig. 2E). Due to the fact that the ELISA for the measurement of IL-1 β is not capable to differentiate between intracellular pro-IL-1 β , which could potentially be released by dead or dying cells, and secreted IL-1 β , we also analyzed the cell death in these monocytes. The proportion of dead monocytes was increased only in cells treated with the pan-caspase inhibitor z-VAD-fmk, both in the LPS/nigericin-treated and the untreated control group (Supporting Information Fig. 2F). It has to be taken into account that higher IL-1 β levels in supernatants of z-VAD-fmk treated cells might be a result of cell death-related pro-IL-1ß release. In addition, we performed experiments in which we induced caspasedependent apoptosis by treating monocytes with staurosporin. We show that the pan-caspase inhibitor z-VAD-fmk was able to protect monocytes from apoptosis as measured by a Tunel assay (Supporting Information Fig. 2G) and by annexin-V staining (Supporting Information Fig. 2H).

GM-CSF/IFN- γ induced cell death in monocytes is mediated by TNF- α

To evaluate the physiological consequence of the GM-CSF/IFN- γ stimulation of monocytes, we treated monocytes for 24 h with GM-CSF \pm IFN- γ and measured the secretion of IL-1 β , TNF- α , and IL-8 in cell supernatants. Concentrations of TNF-a were significantly increased in cell supernatants of GM-CSF/IFN-y cotreated cells, compared with untreated control cells and single treated monocytes (Fig. 3A). Gene expression analyses (qRT-PCR) of selected cytokine genes showed corresponding increases in mRNA levels (data not shown). Interestingly, stimulation of monocytes with TNF- α only did not induce cell death while the costimulation of monocytes with GM-CSF + TNF- α induced necrotic cell death (Fig. 3B). However, the extent of GM-CSF/TNF-a induced cell death of monocytes was significantly lower than in GM-CSF/IFN-y cotreated cells. The data indicate that TNF-α is involved in the process of GM-CSF/IFN-y induced cell death and acts as an additional but not decisive factor. Consequently, TNF-a inhibition reduced GM-CSF/IFN- γ induced cell death (Fig. 3B) as well as the release of IL-8 and IL-1^{\beta} (Fig. 3C). In contrast, IL-1^{\beta} blockade did not significantly inhibit cytokine release and cell death (data not shown). Appropriate isotype controls were used in inhibition experiments



Figure 3. Cytokine release of GM-CSF \pm IFN- γ treated monocytes. (A) Human monocytes from healthy controls were treated with GM-CSF \pm IFN- γ for 24 h or left untreated as a negative control (w/o). Secretion of cytokines was measured in cell supernatants by ELISA. (B) Human monocytes from healthy controls were treated with GM-CSF \pm IFN- ν or TNF- α (10 ng/mL) for 24 h or left untreated as a negative control (w/o). Cell death was measured by double staining with annexin-V and propidium iodide. Cells were cotreated with an antihuman TNF- α (1.25 $\mu g/mL)$ antibody, whereas control cells (w/o) were treated with a matched isotype control. (C) Human monocytes from healthy controls were treated with GM-CSF \pm IFN- γ for 24 h or left untreated as a negative control (w/o). Secretion of IL-8 (black bars) and IL-1β (gray bars) was measured in cell supernatants by ELISA. Cells were cotreated with an anti-human TNF- α (1.25 $\mu\text{g/mL})$ antibody, whereas control cells (w/o) were treated with a matched isotype control. (A-C) Data are shown as mean + SEM (n = 3-12) and are representative of four independent experiments. Significance (refers to unstimulated cells): *p < 0.05; ***p < 0.001.

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and confirmed the specificity of primary antibody binding. Importantly, the effect on IL-1 β (trend of increased release in GM-CSF/IFN- γ treated cells, as well as subsequent reduction with anti-TNF- α) might be a consequence of corresponding effects on cell death and thus release of pro-IL-1 β .

Monocytes surviving GM-CSF/IFN- γ induced cell death are functionally not impaired

To gain insight into the functional relevance of GM-CSF/IFN- γ induced cell death we performed experiments with surviving cells. The response of surviving monocytes to stimuli (LPS) and associated gene expression of cytokines were not reduced (Fig. 4A). ELISA analysis confirmed that cytokine production of surviving GM-CSF/IFN- γ treated monocytes is rather augmented and not impaired (Fig. 4B). Taken together, monocytes that survived GM-CSF/IFN- γ stimulation responded much better to LPS in terms of TNF- α production than untreated monocytes indicating that monocytes were preconditioned by GM-CSF/IFN- γ and amplified their response upon restimulation.

Differences in the cytokine release shown in Figure 4 compared with Figure 3 are most likely due to the fact that the ELISA analysis shown in Figure 3 was performed on cells treated for 24 h with GM-CSF/IFN- γ , whereas the ELISA analysis shown in Figure 4 was performed on cells restimulated for only 4 h with GM-CSF/IFN- γ .

Cathepsin B activation in arthritic joints infiltrated with GM-CSF/IFN- γ double positive T cells

Collectively, our results demonstrate that monocytes respond to costimulation with strong proinflammatory activities in vitro. As a consequence of chronic GM-CSF/IFN-y costimulation, monocytes might undergo activation-dependent cell death as a counter mechanism eliminating overactivated cells. As the enrichment of GM-CSF/IFN-y coexpressing CD4+ T cells in synovial fluid of JIA patients had been described [6], we speculated that GM-CSF/IFN-y induced cell death of monocytes would also occur in vivo in arthritis joints. We thus analyzed cells from patients with active JIA, comparing peripheral blood and synovial fluid. We confirm an accumulation of GM-CSF/IFN-y double positive as well as IL-17 positive CD4+ T cells in arthritic joints (Fig. 5). In contrast to synovial fluid, these T-cell subfractions were not enriched in peripheral blood of JIA patients compared with healthy controls (Fig. 5B). Next we analyzed whether cathepsin B-positive monocytes were enriched in arthritic joints, as cathepsin B expression and activity was identified as the hallmark of GM-CSF/IFN-y induced cell death of monocytes in vitro (Fig. 6A-C). Cathepsin B activity was increased in monocytes isolated from synovial fluid from patients with active arthritis as confirmed by cathepsin B activity assay and immunofluorescence (Fig. 6D and E). Importantly, there were no relevant differences between individual patients (e.g. with versus without immunosuppressive treatment). In addition, we analyzed



Figure 4. Functional properties of surviving cells. (A and B) Human monocytes from healthy controls were treated for 24 h with GM-CSF + IFN- γ or left untreated as controls (w/o) and allowed to rest for 16 h in tissue culture plates to remove dead cells. Adherent monocytes were detached and restimulated for 4 h with GM-CSF ± IFN- γ or LPS. The gene expression of proinflammatory cytokines of surviving monocytes upon restimulation was evaluated by qPCR (A) and confirmed by ELISA (B). (A and B) Data are shown as mean + SEM (n = 3) and are representative of three independent experiments. Significance (refers to control cells [w/o]): *p < 0.05.

the GM-CSF + IFN- γ induced cell death in peripheral blood monocytes from JIA patients and could confirm that also peripheral blood monocytes showed a higher cell death ratios when cotreated with GM-CSF/IFN- γ (Supporting Information Fig. 3). The GM-CSF/IFN- γ induced cell death might be a relevant response mechanism of monocytes in arthritic joints that are enriched with GM-CSF/IFN- γ coexpressing T cells.

Discussion

GM-CSF can elicit different synergistic responses in human monocytes depending on the identity of the costimulating cytokine. A strong synergistic activation of human monocytes by GM-CSF and IFN- γ resulting in release of TNF- α has been established more than two decades ago [13]. It was further shown that GM-CSF and IFN- γ stimulation can override the endotoxin tolerance in LPS-primed monocytes [14, 15]. Furthermore, it has been shown that during experimental autoimmune encephalomyelitis, Th17 cells expressing GM-CSF and/or IFN- γ are highly enriched in myelin oligodendrocyte glycoprotein-specific T cells in the central nervous system and can induce disease independently of Th1 cells [14-16]. It has also been demonstrated that T cell-derived GM-CSF and IFN-y exert unique and synergistic immunostimulatory effects on host macrophages [14, 15]. As our knowledge expands regarding innate immune functions either in response to pattern recognition during host defense or due to cytokine-induced activation during Th1/Th17-driven autoimmunity, also counter mechanisms get into the focus of research. If the above-mentioned processes

would escape regulation, the result could be an indefinite selfamplifying perpetuation of immune responses, e.g. during inflammatory arthritis.

Recently, Th17 plasticity has been identified as a key driver of GM-CSF/IFN- γ production within the arthritic joint in JIA. Here, we confirm the enrichment of T cells that can drive inflammation by coexpressing these potent cytokines in inflamed joints. While we reproduce that simultaneous stimulation of monocytes with GM-CSF and IFN-y results in strong cell activation, our data demonstrate that cell death of human monocytes could represent a significant response mechanism of monocytes both in vitro and in vivo. The elimination of hyperactivated monocytes could represent a process that is capable of downtuning cross-activation between autoreactive T cells and myeloid cells in the context of autoimmune reactions, which may be an important function during the attenuation of autoimmune diseases. AICD of T cells is an important mechanism for maintaining self-tolerance, but AICD of monocytes has been neglected to date [17-19]. However, there is a tight regulation balancing survival versus death signals of monocytes [20-22].

We provide data on infection-independent but activationdependent cell death in monocytes. We found that the deletion of GM-CSF/IFN- γ activated monocytes involves a cell death pathway that is caspase-independent but cathepsin B-mediated and has morphological characteristics of necrosis. We furthermore demonstrate that GM-CSF/IFN- γ induced cell death of monocytes was dependent on the presence of TNF- α and on cathepsin B. Cathepsin B was already shown to be involved in TNF- α induced cell death of fully differentiated macrophages, vascular



Figure 5. IL-17 positive as well as IFN- γ /GM-CSF double positive CD4⁺ T cells in SFMCs of JIA patients. SFMCs and healthy control or JIA PBMCs were isolated from synovial fluid or blood. (A) Gating strategy for intracellular FACS measurement is shown for one SFMC example. (B) Scatter plots for all intracellular cytokine measurements and shown are the mean levels (central horizontal line). Significance: **p < 0.001, **p < 0.001. Data shown are representative of 20 healthy controls and 12 patients with JIA as indicated and from three independent experiments.

endothelial cells, as well as hepatocytes [23-25]. Here, we show that TNF- α is also involved in cathepsin B-mediated cell death of monocytes. It is conceivable that excessive activation of monocytes by GM-CSF, IFN- γ , and TNF- α activates a more complex signaling cascade that involves multiple mediators. As a canonical consequence, however, the resulting bioenergetics exhaustion may lead to the described cell death of monocytes. Monocytes play an important role during the early phase of immune responses, e.g. during host defense. However, counter mechanisms are important to prevent excessive inflammation and autoimmunity leading to chronic disease such as inflammatory arthritis. Thus, GM-CSF/IFN-y induced cell death of monocytes might be beneficial in maintaining homeostasis and self-tolerance in the immune system. In the present study we show that this form of necrotic, proinflammatory monocyte cell death might act as a double-edged sword, which on one hand can further augment acute immune activation but on the other hand also prevent chronic hyperactivation of the immune system by the elimination of monocytes. The latter could possibly prevent further cell differentiation (e.g. DCs), cell polarization, and presentation of self-antigen. We suggest that the GM-CSF/IFN- γ induced cathepsin B-mediated cell death in approx. 30% of monocytes might represent modulatory counter mechanisms during inflammatory arthritis in order to limit excessive inflammation given that our data suggest that remaining/surviving monocytes retain their functionality and that their cell responses upon restimulation (LPS) are even amplified. In agreement with this, we have previously shown that monocytes which were stimulated with GM-CSF responded much better to LPS in

terms of TNF- α and IL-1 β production than untreated monocytes. This indicates that monocytes were preconditioned by GM-CSF alone and amplified their response upon restimulation [26]. Interestingly, it has previously been suggested that resolution of inflammation in the course of a healthy immune response is aided by the unperturbed killing of activated monocytes with inflammatory potential by responder T cells. This supports our hypothesis that the ensuing reduction in the number of proinflammatory monocytes/macrophages may help ensure inflammation is selflimiting and this might be an important step in regulating inflammatory response [12]. Another recent study indicates that monocytes from patients with neonatal-onset multisystem inflammatory disease exposed to LPS exhibit morphologic features of cathepsin B-dependent and caspase 1-independent cell death consistent with pyronecrosis [27]. The authors suggest that the necrotic cell death associated with pyronecrosis and the resulting substantial inflammation may play an important role in the pathogenesis of neonatal-onset multisystem inflammatory disease.

Limitations of our study include that it is methodically challenging to analyze live and dying cells/monocytes separately. Although we address inflammatory cell responses, the further differentiation of effects in surviving and dying cells is certainly a complex issue. It remains a question for further studies whether cells responding to costimulation with GM-CSF and IFN- γ by cytokine release are the ones that die. Furthermore, we performed additional experiments on different human peripheral blood monocyte subpopulations (CD14⁺, CD16⁺, total monocytes). Stimulation of the different monocytes subpopulations with GM-CSF + IFN- γ increased cell death particularly in nonclassical CD16 positive monocytes



Figure 6. Cathepsin B activity in monocytes. (A) For determination of cathepsin B activity (arrows) by immunofluorescence microscopy, human monocytes from healthy controls were cultured in chamber slides. After addition of the cathepsin B-specific substrate reagent MR-(RR)₂ cells were either left untreated or stimulated with GM-CSF + IFN- γ for 4, 8, 12, or 24 h and the nuclei were counterstained with DAPI. (B) Quantitative fluorescence densitometry was used to evaluate MR-(RR)₂ fluorescence. For each condition, total cell fluorescence of 35–50 human monocytes from healthy controls was analyzed. (C) Cathepsin B in cell culture supernatants (SN) of human monocytes from healthy controls was measured by ELISA after 4, 8, 12, or 24 h. (D) Isolated PBMCs and SFMCs of one exemplary JIA patient were stained for cathepsin B activity (arrows) using the substrate MR-(RR)₂. (E) Cathepsin B activity in monocytes was measured by flow cytometry in PBMCs of healthy controls or PBMCs and SFMCs of JIA patients. Shown is the MR-(RR)₂ fluorescence intensity (MFI) gated on CD14⁺ monocytes. (A and D) Scale bar: 20 µm. Images are representative of three independent experiments. (B, C, and E) Data are shown as mean + SEM (B: n = 35-50; C: n = 6; E: n = 4-12 as indicated) and are representative of three independent experiments. Significance was determined by Kruskal–Wallis test (B and E) and Mann–Whitney U test (C): *p < 0.05; **p < 0.01; ***p < 0.00; n.s.: not significant.

(data not shown). Due to the observed high spontaneous cell death in untreated bead-purified monocytes and an additional cell death effect exclusively in CD16 positive monocytes, we can only speculate that CD16 positive monocytes are more susceptible to GM-CSF/IFN- γ induced cell death. However, the detailed GM-CSF/IFN- γ induced effects on different monocytes subpopulations are beyond the scope of the present study and remain to be elucidated in future studies.

Nevertheless, we propose that GM-CSF/IFN- γ induced cell death of monocytes is a means to prevent chronic (smoldering) inflammatory reactions that could facilitate autoimmune arthritis long term. A definite resolution of inflammatory immune responses is critical for the prevention of chronic inflammatory arthritis. Autoimmune reactions may be triggered if inflammation is not resolved but dysregulated during the interaction of T cells with antigen-presenting cells. Freshly recruited monocytes, in particular, may respond to the milieu of cytokine signals generated by effector T-cell subsets typically driving autoimmunity [1, 2, 5, 6]. We demonstrate that besides T cells expressing GM-CSF/IFN- γ being enriched in affected joints during JIA,

also responder monocytes are present that become hyperactivated involving processes that include early activation-dependent cell death as a means of regulation. We cannot exclude that simultaneous activation of monocytes by GM-CSF/IFN- γ will also influence later events during monocyte differentiation into either anti-inflammatory macrophages or tolerogenic DC populations as described [28]. Nevertheless, we conclude that monocytes rescued from cell death during hyperactivation can possibly develop into competent macrophages with key functions during later phases of inflammation and healing. We thus propose that GM-CSF/IFN- γ induced cell death of monocytes might serve as an important modulatory mechanism during inflammatory arthritis.

Materials and methods

Patients and controls

Samples studied were from 15 children with active JIA (12 with oligoarticular disease, three with polyarticular disease) who met

the International League against Rheumatism criteria [29] and 11 adult healthy controls as well as ten children healthy controls (median age 6.0). Eleven of the JIA patients were female and four were male; the median age was 13.7 years. Nine patients received immunosuppressive medication: methotrexate (n = 6), sulfasalazine (n = 1), antitumor necrosis factor alpha (n = 2). Thirteen patients were also on medication with a nonsteroidal anti-inflammatory drug. The study was approved by the ethics committee of the University of Münster, Germany (reference number 2014-273-b-s), and full informed consent was obtained from patients/parents and control subjects.

Isolation of peripheral blood mononuclear cells, synovial fluid mononuclear cells, and monocytes

Peripheral blood from individual healthy donors was obtained from the Department of Transfusion Medicine at the University Hospital Münster, Germany. Peripheral blood leukocytes were taken from donors by leukapheresis and isolated by Ficoll-Paque and subsequent Percoll (Pharmacia, Freiburg, Germany) densitygradient centrifugation to >90% purity (Supporting Information Fig. 1) as previously described [30]. Monocytes were cultured (1×10^6 cells/mL) in hydrophobic teflon bags (Heraeus, Hanau, Germany) in McCoy's 5a medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 200 IU/mL penicillin, 100 µg/mL streptomycin, and $1 \times$ nonessential amino acids (all from Biochrom, Berlin, Germany) and allowed to rest for 24 h prior to stimulation.

Isolation of healthy control and JIA PBMCs from whole blood samples and JIA synovial fluid mononuclear cells (SFMCs) from synovial fluid was performed by Ficoll-Paque density gradient centrifugation (Pharmacia). For SFMC isolation, synovial fluid was pretreated with hyaluronidase (20 U/mL, Sigma, Taufkirchen, Germany) and synovial debris was removed. To further enrich monocytes from PBMCs and SFMCs, the EasySep human monocyte enrichment kit was used according to the manufacturer's protocol (STEMCELL Technologies, Cologne, Germany).

Determination of cell death and intracellular staining by flow cytometry

One of the early features of apoptosis is the loss of cell membrane asymmetry/integrity and the externalization of phosphatidylserine, which was measured by flow cytometric staining with FITC-conjugated annexin-V (BD Pharmingen, Heidelberg, Germany) and propidium iodide (PI, Sigma). The formation of fragmented DNA in apoptotic nuclei was assessed by the method of Nicoletti et al. [31]. For detection of cell surface molecules, flow cytometry was performed as described earlier [32]. All intracellular stainings were performed using the transcription factor staining buffer set (eBioscience, San Diego, CA, USA). Monoclonal antibodies used are: CD11b (M1/70), CD4 (RPA-T4), IL-17a (BL168), GM-CSF (BVD2-21C11), IFN- γ (4S.B3; all from BioLegend, San Diego,

CA, USA). Flow cytometry measurements were performed using a FACScanto A (Becton Dickinson, Heidelberg, Germany). Collected data were analyzed by FlowJo software (version 10.0.8, FlowJo LLC, Ashland, OR, USA).

Monocyte stimulation and cell death assays

Monocytes were stimulated for different time periods with GM-CSF (10 ng/mL, MP Biomedicals, Aurora, CO, USA) \pm IFN- γ (25 ng/mL; Immunotools, Friesoythe, Germany) or left untreated. Cells were precultured for 1 h in the presence of irreversible protease inhibitors: broad-spectrum caspase inhibitor z-VAD-fmk (Z-Val-Ala-DL-Asp-fluoromethylketone, 50 µM, Bachem, Heidelberg, Germany), caspase-1 inhibitor z-YVAD-fmk (Z-Tyr-Val-Ala-DL-Asp-fluoromethylketone, 50 µM, Bachem), and also cathepsin B inhibitor z-FA-fmk (50 µM, Merck, Darmstadt, Germany). Likewise, necrostatin-1 (15 µM, Sigma) was used as an allosteric RIP1 kinase inhibitor, which inhibits death receptor-induced necroptosis [33]. In additional experiments, cells were cotreated for 24 h with anti-human IL-1 β (1.25 μ g/mL, eBioscience, Frankfurt/Main, Germany) and anti-human TNF- α (1.25 µg/mL, eBioscience, Frankfurt/Main, Germany) antibodies. To exclude unspecific fc-receptor mediated activation of monocytes, corresponding antibody isotype controls (1.25 µg/mL mouse IgG1) were used. For restimulation experiments, monocytes were treated for 24 h as described above and allowed to rest for 16 h in tissue culture plates to remove dead cells. Adherent monocytes were detached with 0.05% trypsin/0.02% EDTA in phosphate-buffered saline solution and restimulated for 4 h with GM-CSF + IFN- γ , IFN- γ , or LPS (manufacturers and concentrations as described above). Cell supernatants and RNA were stored at -20°C for analysis of cytokine secretion and qRT-PCR.

Caspase activity assays

For evaluation of caspase activity, CaspGlow Fluorescein Active Caspase 3, 8, and 9 kits were used according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Cells treated for 4 h with 400 nM staurosporine (Sigma–Aldrich, Steinheim, Germany) were used as positive control. Finally, cells were washed and further stained for annexin-V and 7AAD and analyzed by flow cytometry using a FACSCanto A (Becton Dickinson).

Evaluation of cell death by immunofluorescence microscopy

Monocytes (2 × 10⁵) were cultured in fibronectin coated 8-well Nunc Lab-Tek chamber slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) and were either left untreated or stimulated with GM-CSF, IFN- γ or GM-CSF + IFN- γ for 4, 8, 12, or 24 h at 37°C/5% CO₂. After incubation, cells were washed with AnnexinV binding buffer (eBioscience, San Diego, CA, USA) and cell death was detected by using phosphatidylserine sensor Apopxin Green Indicator (Abcam, Cambridge, UK) and propidium iodide staining. Cells were washed; fixed with formaldehyde (4%, 4°C, overnight) and nuclei counterstained with Hoechst 33258 (Thermo Fisher Scientific Inc.). After mounting, specimens were visualized using an AxioObserver.Z1 microscope with EC Plan-Neofluar objectives and Axiovision 4.8 software (Carl Zeiss, Jena, Germany).

Cathepsin B activity measurement

For determination of cathepsin B activity by immunofluorescence microscopy, 1×10^5 monocytes were cultured in fibronectin coated 8-well Nunc Lab-Tek chamber slides (Thermo Fisher Scientific Inc.). After addition of the cathepsin B-specific substrate reagent MR-(RR)₂ (ImmunoChemistry Technologies, Bloomington, MN, USA) according to the manufacturer's recommendations cells were either left untreated or stimulated with GM-CSF + IFN- γ for 4, 8, 12, or 24 h at 37°C/5% CO_2 . After incubation cells were washed and fixed with formaldehyde (4%, 4°C, overnight), permeabilized with Triton X-100 (0.1%, RT, 15 min), and nuclei counterstained with DAPI. In addition, PBMCs and SFMCs of JIA patients with active disease were used without stimulation. After mounting, specimens were visualized using an AxioObserver.Z1 microscope with an EC Plan-Neofluar 40× objective and Axiovision 4.8 software (Carl Zeiss). We used quantitative fluorescence densitometry to evaluate the cathepsin B activity in a nonbiased semiautomated fashion. All densitometry readings were performed at \times 400 magnification with exposure times of 3000 ms for detection of MR-(RR)2 fluorescence. For each condition, total cell fluorescence of 35-50 monocytes was analyzed using ImageJ software (version 1.47q, NIH, USA).

For measuring cathepsin B activity by FACS, PBMCs and SFMCs were incubated for 1 h with MR-(RR)₂ and subsequently counterstained for CD14. Activity was measured using a FACSCanto A as described elsewhere [34]. Cathepsin B in cell culture supernatants was measured using the Cathepsin B Human ELISA Kit (Abcam) according to the manufacturer's recommendations.

Cytokine measurement

TNF- α , IL-1 β , and IL-8 concentrations in cell culture supernatants were determined by ELISA (OptEIA ELISA kits, BD Pharmingen).

Quantitative real-time PCR

Expression of selected genes in human monocytes of healthy donors was confirmed by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) as described previously [35]. PCRs were performed and measured on an ABI Fast Real-Time 7900HT system (Life Technologies, Darmstadt, Germany). The relative expression was calculated using RPL as endogenous housekeeping control gene. Primers used were as follows: TNF- α forward 5'-CTT CTC GAA CCC CGA GTG AC-3', reverse 5'-TGA GGT ACA GGC CCT CTG ATG-3'; IL-1 β forward 5'-GCG GCC AGG ATA TAA CTG ACT TC-3', reverse 5'-TCC ACA

TTC AGC ACA GGA CTC TC-3'; IL-8 forward 5'-CTT GTT CCA CTG TGC CTT GGT T-3', reverse 5'-GCT TCC ACA TGT CCT CAC AAC AT-3'; and RPL forward 5'-AGG TAT GCT GCC CCA CAA AAC-3', reverse 5'-TGT AGG CTT CAG ACG CAC GAC-3'.

Statistics

Data are expressed as mean \pm SEM and were assessed using the Mann–Whitney *U* test except when stated otherwise. Values of p < 0.05 were considered statistically significant. All calculations were performed using SPSS version 14 (SPSS Inc., Chicago, IL, USA). Graphs were produced in GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).



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Abbreviations: AICD: activation-induced cell death · JIA: juvenile idiopathic arthritis · SFMCs: synovial fluid mononuclear cells

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Supplementary Figure 1



Supporting Information Figure 1: Purity of isolated monocytes

Human monocytes from healthy controls were isolated from PBMCs generated by Ficoll density-gradient centrifugation and subsequent Percoll density-gradient centrifugation. (Left and middle panel) Purity of resulting monocytes is shown together with the gating strategy in a representative dot plot showing CD14 expression on the y-axis and CD16 expression on the x-axis. (Right panel) Contaminating cells are shown for isolated monocytes in an FSC and SSC dot plot showing linage marker expressing cells overlaid and percentage of positive cells (frequency of parent).

Supplementary Figure 2



Supporting Information Figure 2: Inhibitor functionality

(A) To evaluate the functionality of the cathepsin B inhibitors z-FA-fmk (50 μ M) and CA074 (100 μ M) cells were pre-treated (1 hour) with either one of both inhibitors and subsequently stimulated for 3 hours with nigericin (20 μ M) or were left untreated as control. Cathepsin B activity in GM-CSF + IFN- γ (6 hours) costimulated monocytes and untreated controls is shown. (B) Cathepsin B activity was determined by measuring the specific substrate reagent MR-(RR)₂ fluorescence in a plate reader system (n=6). Inhibition and cathepsin B activity measurement were performed as described in A. (C) To evaluate necrostatin efficiency RIPK1 mediated cell death was induced by stimulating monocytes for 6 hours with LPS and z-VAD-fmk. Inhibition of RIPK1 by necrostatin or as negative control with other inhibitors were performed by treating monocytes for 1 hour in advance with the specific inhibitors. Shown is the percentage of dead cells as evaluated by flow cytometry using annexin-V and 7-AAD staining (n=3). (D) Shows the viability of the same cells used in C and determined by using a fluorescent viability dve. (E) To evaluate the functionality of the caspase 1 inhibitor z-vVAD-fmk cells were pretreated (1 hour) with the specific inhibitor or other inhibitors as control. The release of IL-1 β was measured by ELISA in supernatants of monocytes in which caspase 1 activity was induced by treating the cells for 6 hours with LPS (1 µg/mL) and for the final 3 hours with nigericin (1 µM). Untreated cells served as a control (n=3). (F) To test for unspecific intracellular pro-IL-1ß release by dying cells the percentage of dead cells was evaluated by Annexin-V and 7-AAD staining (shown are single and double positive cells). The functionality of the pan-caspase inhibitor z-VAD-fmk was evaluated by induction of caspase-depended apoptosis by staurosporine in monocytes. Apoptosis was measured by Tunel assay (n=3) (G) and by measuring annexin-V positive cells by flow cytometry (n=3) (H). Data are shown as mean + SEM (n=3) and are representative of 3 independent experiments. Statistical significance was determined by Mann Whitney U test. If not indicated otherwise * refers to control cells: *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary Figure 3



Supporting Information Figure 3: Cell death in peripheral blood monocytes of JIA patients

Monocytes were treated with GM-CSF (10 ng/mL) \pm IFN- γ (25 ng/mL) for 24 hours or left untreated as a negative control (w/o). Cell death was measured by double staining with annexin-V and 7-AAD and shown is the percentage of total dead cells for each individual JIA patient (inactive disease, n=3; active disease, n=3). Statistical significance was determined by Mann Whitney U test: **P < 0.001.

Supplementary Materials and Methods

Determination of peripheral blood monocyte purity

Purity of isolated peripheral blood monocytes was validated using flow cytometry and counterstaining for CD14 (61D3, eBioscience, San Diego, CA, USA) and CD16 (eBioCB16, eBioscience) but also by staining for potentially contaminating cells with linage defining markers such as CD56 (HCD56, Biolegend, San Diego, CA, USA), CD3 (UCHT1, Biolegend), CD66b (G10F5, eBioscience), CD19 (HIB19, Biolegend), CD20 (2H7, Biolegend). Flow cytometry measurements were performed using a FACScanto A (Becton Dickinson, Heidelberg, Germany). Collected data were analyzed by FlowJo software (v10.0.8, FlowJo LLC, Ashland, OR, USA).

Patients

Blood samples from 6 patients with juvenile idiopathic arthritis (JIA) who met the International League Against Rheumatism criteria were analyzed (inactive disease, n=3; active disease, n=3). Disease activity was assessed using the Juvenile Arthritis Disease Activity Score (JADAS), a composite disease activity score for JIA. Patients were on immunosuppressive medication (non-steroidal antirheumatics, n=1; anti-TNF α , n=2; anti-IL-1 β , n=3) and the median age was 10 years. The study was approved by the ethics committee of the University of Münster, Germany (reference number 2014-273-b-s) and fully informed consent was obtained from patients/parents and control subjects.

Monocyte isolation and stimulation

To isolate peripheral blood monocytes from 5 mL EDTA whole blood samples of patients with JIA the EasySepTM direct human monocyte isolation kit was used according to the manufacturer's protocol (STEMCELL Technologies, Cologne, Germany). Monocytes were cultured (1x10⁶ cells/mL) in hydrophobic teflon bags (Heraeus, Hanau, Germany) in McCoy's 5a medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 200 IU/mL penicillin, 100 μ g/mL streptomycin and 1x non-essential amino acids (all from Biochrom, Berlin, Germany) and were left untreated or stimulated with GM-CSF (10ng/mL; Peprotech, Hamburg, Germany) ± IFN- γ (25ng/mL; Immunotools, Friesoythe, Germany) for 24 hours.

Inhibitor studies

Monocytes were stimulated for 6 hours with GM-CSF (10 ng/mL, Peprotech, Hamburg, Germany) \pm IFN- γ (25 ng/mL; Immunotools, Friesoythe, Germany) or left untreated. Monocytes were treated for 3 hours with 20 μ M nigericin (Sigma-Aldrich, Taufkirchen, Germany) to induce cathepsin B activity. Some monocytes were stimulated for 6 hours with LPS (1 μ g/mL, Sigma-Aldrich, Taufkirchen, Germany) and z-VAD-fmk (Z-Val-Ala-DL-Asp-fluoromethylketone, 50 μ M, Bachem, Heidelberg, Germany) to induce RIP1 kinase mediated cell death. The inflammasome was induced in monocytes by treating cells for 6 hours with LPS (1 μ g/mL) and for the final 3 hours with nigericin (1 μ M). Induction of caspase-depended apoptosis in monocytes was induced with staurosporine (2.5 μ M, Sigma-Aldrich, Taufkirchen, Germany). For inhibitor studies cells were pre-cultured for 1 hour in the presence of the following inhibitors: broad-spectrum caspase inhibitor z-VAD-fmk (50 μ M), caspase-1 inhibitor z-YVAD-fmk (Z-Tyr-Val-Ala-DL-Asp-fluoro-methylketone, 50 μ M, Bachem, Heidelberg, Germany), cathepsin B inhibitors z-FA-fmk (50 μ M, Merck, Darmstadt, Germany), and/or CA074 (100 μ M Apexbio, Houston, TX, USA). Necrostatin-1 (15 μ M, Sigma-Aldrich, Taufkirchen, Germany) was used as an allosteric RIP1 kinase inhibitor.

Assessment of cell death and viability

Cell death was evaluated in 1x10⁵ monocytes by flow cytometric co-staining of APC-conjugated annexin-V and 7-AAD (both Biolegend, San Diego, CA, USA) and analyzed using a FACScanto A (Becton Dickinson, Heidelberg, Germany). DNA fragmentation was analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using the Cell Meter[™] TUNEL apoptosis assay kit (ATT Bioquest®, Sunnyvale, CA, USA) according to the manufacturer's recommendations. Viability of cells was measured by using the MultiTox-Glo Multiplex Cytotoxicity Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cathepsin B activity measurement

For determination of cathepsin B activity in a fluorescence ELISA plate reader, $2x10^5$ /mL monocytes were placed into a black 96 well plate (Greiner Bio-One, Frickenhausen, Germany) and the cathepsin B specific substrate reagent MR-(RR)₂ (ImmunoChemistry Technologies, Bloomington, MN, USA) was added for 1 hour according to the manufacturer's recommendations. Monocytes without substrate served as control. Substrate fluorescence was read in an Infinite m200 fluorescence ELISA plate reader (ex: 590nm em: 620nm, Tecan, Groeding, Austria).