

RESEARCH ARTICLE

Apoptotic-cell-derived membrane microparticles and IFN- α induce an inflammatory immune response

Anna Niessen*, Petra Heyder, Stefan Krienke, Norbert Blank, Lars-Oliver Tykocinski, Hanns-Martin Lorenz and Martin Schiller

ABSTRACT

A dysregulation in the clearance of apoptotic material is considered a major pathogenetic factor for the emergence of autoimmune diseases. Apoptotic-cell-derived membrane microparticles (AdMPs), which are released from the cell surface during apoptosis, have been implicated in the pathogenesis of autoimmunity. Also of importance are cytokines, such as interferon- α (IFN- α), which is known to be a major player in patients with systemic lupus erythematosus (SLE). This study investigates the combined effect of AdMPs and IFN- α on professional phagocytes. In the presence of IFN- α , phagocytosis of AdMPs by human monocytes was significantly increased in a dose-dependent manner. The combination of AdMPs and raised IFN- α concentrations resulted in an increase in the secretion of pro-inflammatory cytokines and an upregulation of surface molecule expression involved in antigen uptake. In addition, macrophage polarisation was shifted towards a more inflammatory type of cell. The synergism between IFN- α and AdMPs seemed to be mediated by an upregulation of phosphorylated STAT1. Our results indicate that IFN- α , together with AdMPs, amplify the initiation and maintenance of inflammation. This mechanism might especially play a crucial role in disorders with a defective clearance of apoptotic material.

KEY WORDS: Apoptosis, Cell blebbing, Clearance, Interferon- α , Membrane microparticle, Systemic lupus erythematosus

INTRODUCTION

To date, the pathogenesis of autoimmunity and auto-inflammation is only partially understood. In systemic lupus erythematosus (SLE), major pathogenetic factors are an increased rate of apoptosis and a defective clearance of apoptotic cells. Human SLE is an autoimmune disorder characterised by high concentrations of circulating autoantibodies. These antibodies target nuclear antigens as well as DNA, structures that are usually well-hidden from the host's immune system. Physiologically, programmed cell death ensures the swift and non-inflammatory removal of these intracellular antigens. During apoptosis, fragmentation of intracellular substrates is mediated by caspases (Earnshaw, 1995; Schiller et al., 2008). However, the process of protein cleavage by caspases also creates numerous neo-epitopes. This increases the risk of eliciting autoimmune responses if the apoptotic material is not deposited and removed in an anti-inflammatory manner. In this context, subcellular membrane vesicles that are released from apoptotic cells might be of major relevance. Dynamic cell blebbing and the release of apoptotic-cell-derived membrane microparticles (AdMPs) is a

hallmark feature of apoptosis, and is thought to be the result of cytoskeletal reorganisation and contraction (Lubkov and Bar-Sagi, 2014; Mills et al., 1998; Ndozangue-Touriguine et al., 2008). Importantly, microparticles formed during programmed cell death (AdMPs) differ structurally from those vesicles released from activated cells. Furthermore, they are completely different from another type of subcellular vesicles, the so-called exosomes. The latter accumulate within a compartment, the multivesicular body (MVB), and are released directly into the extracellular space, either constitutively or upon stimulation, by fusion of the MVB with the plasma membrane (exocytosis). In contrast to this, AdMPs and membrane microparticles shed from viable cells are released directly from the cell surface by budding of the cellular membrane (Sebbagh et al., 2001).

Importantly, we and other groups have shown that AdMPs contain antigens that are relevant autoantibody targets in autoimmune diseases (Radic et al., 2004; Reich and Pisetsky, 2009; Schiller et al., 2008). In addition, molecules known to be involved in immune reactions, such as high mobility group box protein 1 (HMGB1), have been detected within apoptotic microparticles (Pisetsky, 2014; Schiller et al., 2013; Spencer et al., 2014). Defects in clearance of apoptotic material are considered a major pathogenetic factor in the development of SLE, and high levels of circulating apoptotic material have been found in many patients. Moreover, AdMPs have recently been implicated in potential immune regulatory and auto-adjuvant functions during immune responses. These data led to our hypothesis that AdMPs constitute one of the missing links between the dysregulated process of apoptosis and the access of immune cells to nuclear autoantigens in the pathogenesis of SLE.

Another important factor involved in the pathogenesis of SLE is the type-I interferon interferon- α (IFN- α). Under physiological conditions, IFN- α is produced by plasmacytoid dendritic cells (pDCs) in response to microbial invasion. The secretion of IFN- α is mediated by stimulation of toll-like receptors (TLRs) by single-stranded RNA (ssRNA) or CpG-rich double-stranded DNA (dsDNA). In SLE, however, the production of IFN- α seems to be triggered by interferogenic immune complexes that consist of nucleic acids and autoantibodies (Kassim et al., 2013; Lövgren et al., 2004; Rönnblom, 2011). Serum levels of IFN- α correlate with disease activity, as well as with levels of dsDNA antibodies (Becker-Merok et al., 2013). Furthermore, patients receiving treatment with IFN- α have been observed to develop autoimmune symptoms and transient autoantibody formation has been detected in their sera. During the past years, blockage of IFN- α has therefore been focus of pharmacological research (Petri et al., 2013). Only recently, our group was able to prove a direct link between the release of AdMPs and IFN- α secretion, showing that AdMPs can trigger the secretion of IFN- α from plasmacytoid dendritic cells (Schiller et al., 2012). These data provide a further link between AdMPs and characteristic features of SLE.

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Received 19 September 2014; Accepted 27 May 2015

Therefore, we aimed to evaluate in more detail the biological properties of AdMPs and their effects on professional immune cells.

In this study, we investigated the effect of AdMPs on professional phagocytes in the presence of raised IFN- α concentrations. We examined changes in phagocytosis and cytokine secretion patterns as well as surface molecule expression by monocytes and macrophages in both SLE patients as well as in healthy donors. We also looked at differences in the uptake of AdMPs by monocytes and compared it with that in whole apoptotic cells or necrotic cells. We observed that AdMPs in combination with IFN- α established a generally pro-inflammatory setting through an increase in pro-inflammatory cytokine secretion, as well as upregulation of co-stimulatory molecules. AdMPs and IFN- α caused a shift in macrophage polarisation towards a more inflammatory type of cell. The observed effect was exclusive for AdMPs, as no similar changes were observed for either whole apoptotic or necrotic cells.

RESULTS

Phagocytosis of AdMPs by monocytes shows a dose-dependent increased in the presence of IFN- α

AdMPs have recently been implicated as regulators of inflammatory reactions. As patients with SLE have been found to have both increased amounts of circulating apoptotic material as well as pathologically high levels of IFN- α , we were interested in determining the effect of raised IFN- α on the uptake of apoptotic material (apoptotic cells and AdMPs) by professional phagocytes.

For this purpose, monocytes from normal healthy donors (NHDs) were isolated and co-incubated with CFSE-labelled AdMPs from polymorphonuclear neutrophils (PMNs) of the same donor in the presence or the absence of IFN- α . A flow cytometric phagocytosis assay was performed. Analysis of the mean fluorescence intensity (MFI) gating on the phagocytes showed a significant increase of AdMP engulfment in the presence of IFN- α , compared to cells that did not receive this stimulation (Fig. 1A).

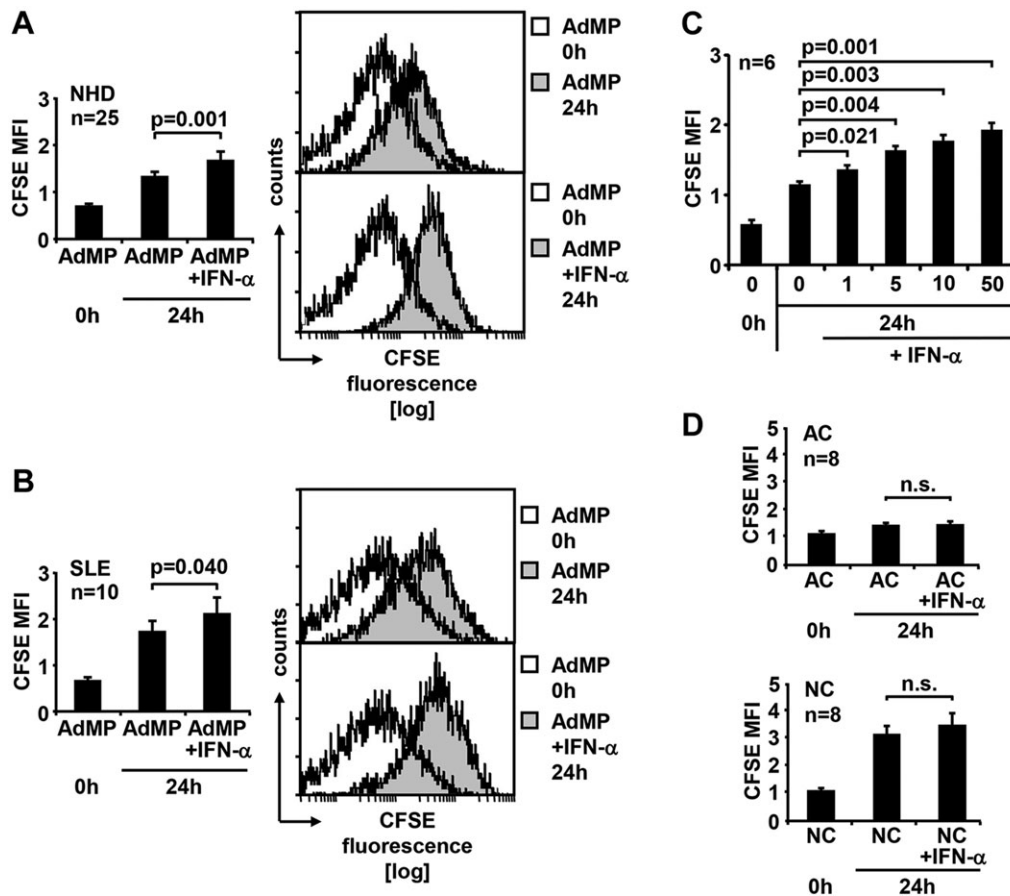


Fig. 1. Phagocytosis of autologous AdMPs by monocytes is increased in the presence of IFN- α . (A) CFSE-stained AdMPs obtained from PMNs of healthy donors (NHDs) were co-incubated with monocytes from the same donor in the presence (AdMP+IFN- α) or absence (AdMP) of 5 ng/ml IFN- α . Phagocytosis was measured by flow cytometry. The left graph shows results obtained from 25 independent experiments. An increase in MFI indicates an engulfment of AdMPs by monocytes. Engulfment was quantified after 24 h of incubation (24 h). As a control, monocytes were analysed directly after addition of AdMPs (0 h). The histogram on the right shows original data obtained from one representative experiment. (B) Monocytes and AdMPs obtained from SLE patients were analysed in the same way as in A. The left graph shows results obtained from 10 independent experiments. The right histogram shows original data obtained from one representative experiment. (C) Monocytes were co-incubated together with AdMPs in the presence of an increasing concentration of IFN- α (1, 5, 10, or 50 ng). Engulfment of AdMPs was quantified after 24 h of incubation. Monocytes analysed directly after addition of AdMPs served as a control (0 h). Data was obtained from six independent experiments. (D) Engulfment of whole apoptotic and necrotic cells by monocytes is not influenced by IFN- α . Monocytes and CFSE-stained, apoptotic or necrotic PMNs from the same donors were co-incubated. Engulfment of apoptotic cells (AC, left graph) or necrotic cells (NC, right graph) was quantified after 24 h of incubation. Furthermore, the effect of an additional treatment with 5 ng/ml IFN- α was analysed (AC+IFN- α , NC+IFN- α). Monocytes analysed directly after addition of apoptotic or necrotic cells served as a control (0 h). The graphs show results obtained from eight independent experiments. Mean values+s.e.m. are shown, statistical significance was tested by Student's *t*-test, n.s. indicates no significant difference.

In order to determine whether the examined effect is conserved in patients with SLE, we repeated the experiments using monocytes and PMNs obtained from SLE patients. We observed a similar increase in phagocytosis of AdMPs by monocytes in the presence of IFN- α (Fig. 1B). No significant differences were observed between healthy donor cells and SLE patients *in vitro*.

As levels of IFN- α correlate with disease activity, we examined further whether uptake of AdMPs would alter with different IFN- α concentrations. Monocytes from both SLE patients and NHDs were found to engulf AdMPs in a dose-dependent manner, when they were cultured together with AdMPs and increasing concentrations of IFN- α (Fig. 1C). Comparing cells obtained from SLE patients to cells obtained from healthy donors, we did not observe any difference in the amount of engulfed AdMPs (data not shown).

Increase in phagocytosis caused by IFN- α is exclusively observed for AdMPs

AdMPs containing autoantigens as well as potential neo-epitopes are effectively cleared by professional phagocytes and antigen-presenting cells (Fehr et al., 2013; Schiller et al., 2008). Whole apoptotic cells, however, can lose their membrane integrity during later stages of apoptotic cell death and enter a post-apoptotic stage, called secondary necrosis. Both secondary and primary necrotic cells release their content into the extracellular space and subsequently induce an inflammatory reaction. Different responses of antigen-presenting cells to AdMPs, whole apoptotic cells and necrotic cells

have been described (Fehr et al., 2013). We were interested whether the observed effect of IFN- α on phagocytosis of AdMPs was also present if whole apoptotic or necrotic cells were engulfed, or whether this effect was specific for AdMPs. We therefore conducted a set of experiments in which monocytes obtained from healthy donors were co-incubated with whole apoptotic or necrotic PMNs in the presence or absence of IFN- α . In these experiments, the presence of IFN- α did not alter the uptake of either whole apoptotic or necrotic cells (Fig. 1D). Thus, the IFN- α dependent increase in the engulfment of apoptotic material was exclusively observed for isolated AdMPs.

Phagocytosis of AdMPs is dependent on SCARF1

In order to ensure that AdMPs were taken up by monocytes and not merely attached to their cell surfaces, confocal microscopy was performed. CFSE-labelled AdMPs and monocytes were co-incubated for 24 h as described above. Confocal microscopy (Fig. 2A) in fact showed that there was a definite engulfment of the AdMPs by monocytes.

Furthermore, in order to prove the vesicular shape and stability of microparticles isolated from apoptosing cells, AdMPs were harvested and prepared for transmission electron microscopy (TEM). TEM imaging revealed the vesicular shape of AdMPs and proved that stability of the AdMP structure was maintained during the process of purification (Fig. 2B).

Dying cells have been shown to expose so-called 'eat-me' signals on their cell surface, which under normal healthy circumstances

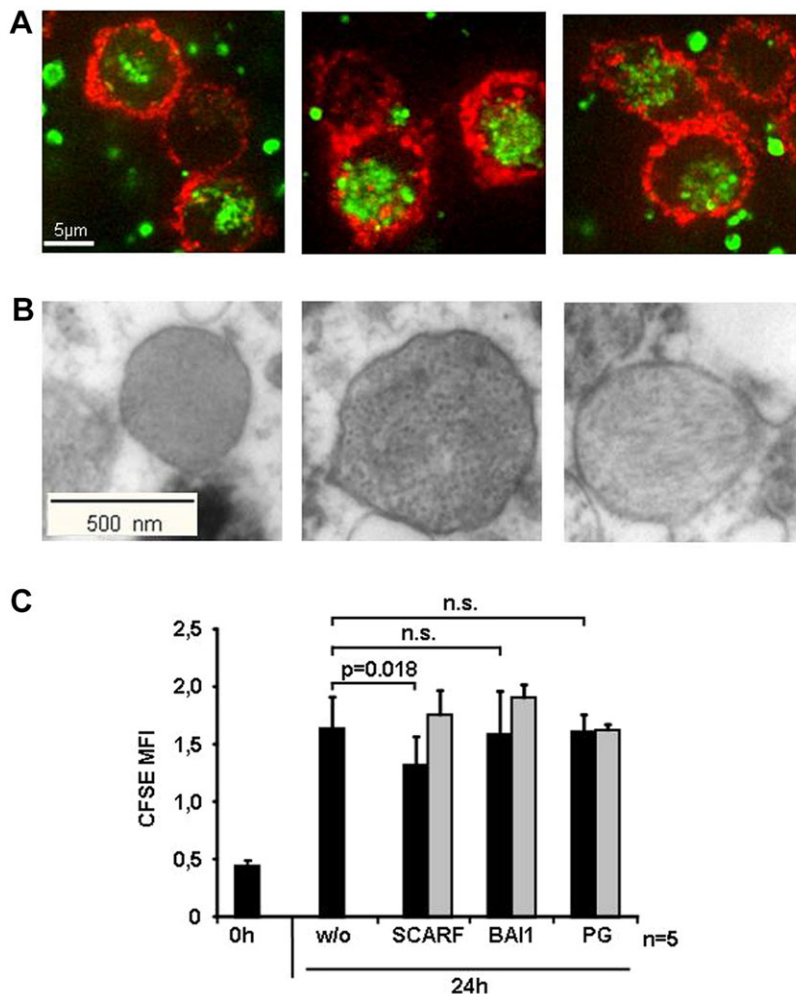


Fig. 2. Engulfment of AdMPs by monocytes is dependent on SCARF1. (A) Monocytes (stained with CD14–phycoerythrin, shown in red) and AdMPs (stained with CFSE, shown in green) obtained from NHDs were co-incubated for 24 h and then analysed by confocal microscopy. Confocal microscopy revealed the intracellular localization of engulfed AdMPs. Three representative sections are shown in the figure. (B) AdMPs were generated from apoptotic T lymphocytes from NHDs and analysed by TEM after 24 h. Representative sections are shown in the figure. (C) Monocytes were incubated with either BAI1-blocking antibody (8 μ g/ml) (BAI1), SCARF1-blocking antibody (0.5 μ g/ml) (SCARF) or 1 μ g/ml polyglobulin (PG) prior to addition of AdMPs (black bars). After 24 h, phagocytosis was then compared to monocytes left untreated (w/o) (black bar). Furthermore, the effect of additional treatment with 5 ng/ml IFN- α was tested (grey bars). Monocytes analysed directly after addition of AdMPs served as a control (0 h). The graphs show results obtained from five independent experiments. Mean+s.e.m. are shown, statistical significance was tested by Student's *t*-test, n.s. indicates no significant difference.

facilitate swift removal of apoptotic cells without inflammatory reactions. The exposure of phosphatidylserine (PtdSer) on the cell surface is a hallmark feature of apoptosis (Fadok et al., 2001) and several factors have been shown to be involved in uptake of apoptotic cells by binding to PtdSer, such as brain-specific angiogenesis inhibitor 1 (BAI1) (Park et al., 2007) and factors of the T-cell immunoglobulin mucin (TIM) family (Kobayashi et al., 2007). In addition, factors such as scavenger receptor 1 (SCARF1; originally called scavenger receptor expressed by endothelial cell 1, SREC-1) have been shown to facilitate phagocytosis of apoptotic cells through PtdSer-independent pathways (Ramirez-Ortiz et al., 2013). Although uptake of apoptotic cells has been focus of intensive research, little is known about the mechanisms of AdMP uptake. In diseases like SLE, where high levels of circulating antibodies occur, an immunoglobulin-dependent uptake might also play a role. We therefore examined possible factors involved in AdMP uptake. Monocytes and CFSE-labelled AdMPs from PMNs of the same NHD were isolated. Monocytes were then incubated with BAI1-blocking antibody, SCARF1-blocking antibody and human polyglobulin reagent each in the presence and absence of IFN- α . After an incubation period of 1 h, AdMPs were added and incubated for 24 h. Phagocytosis was then analysed by flow cytometry. We observed a significant decrease in phagocytosis upon blockage of SCARF1 receptor compared to cells left untreated (Fig. 2C). Interestingly, the addition of IFN- α abrogated the observed inhibition in uptake. In contrast, no significant difference in uptake of AdMPs was seen when BAI1-blocking antibody or polyglobulin was added to the culture (Fig. 2C).

AdMPs and IFN- α synergistically induce the production of pro-inflammatory cytokines

In order to determine whether the uptake of AdMPs alters the functional properties of monocytes, we examined the cytokines released from monocytes that had engulfed AdMPs. Monocytes were incubated for 24 h with AdMPs in the presence or absence of IFN- α . The supernatants were collected and the cytokine content within the supernatant was compared to supernatants from cells either left untreated or from those that received IFN- α only.

We found that the presence of AdMPs as well as IFN- α had a highly significant impact on the secretion of pro-inflammatory cytokines. Upon incubation with AdMPs alone, the secretion of interleukin-6 (IL-6) by monocytes was increased by more than twofold, whereas incubation with IFN- α alone did not cause a relevant increase in IL-6 secretion (Fig. 3A). However, monocytes that were treated with AdMPs as well as IFN- α showed a further significant increase in the secretion of IL-6 (Fig. 3A). Similarly, the amount of tumor-necrosis factor α (TNF- α) secreted by monocytes was increased significantly upon stimulation with AdMPs, and the combination of IFN- α and AdMPs resulted in a further significant increase (Fig. 3B). The pro-inflammatory cytokine IL-8 was released at highest concentrations (Fig. 3C). In all experiments, the amount of IL-8 was increased when monocytes were incubated with AdMPs. Supplementation with IFN- α alone caused only a slight increase in IL-8 secretion. Again, the stimulation of monocytes with AdMPs plus IFN- α caused a further increase in the amount of IL-8. As shown in Fig. 3D, the anti-inflammatory cytokine IL-10 was also found in the supernatants of all four conditions. However, no significant difference in the secretion pattern was seen in the presence of AdMPs or IFN- α .

As we were able to show a reduction in phagocytosis of AdMPs by monocytes upon the addition of SCARF1 blocking antibody (Fig. 2C), we further analysed the secretion of the pro-inflammatory

cytokines IL-6 and TNF- α into the supernatants of these co-cultures after 24 h. We observed an inhibition of the secretion of both cytokines (IL-6, Fig. 3E; TNF- α , Fig. 3F) if SCARF1-blocking antibody was added to the co-culture.

Expression of co-stimulatory cell surface molecules is synergistically increased after stimulation of monocytes by AdMPs and IFN- α

It still remains unclear, how autoantigens that are confined within a plasma membrane become exposed to the immune system of the host in SLE patients. Therefore, we were interested to examine specifically the role of AdMPs – as a reservoir of autoantigens and neo-epitopes – in the regulation of surface markers involved in antigen uptake, processing and presentation. Thus, the expression of cell surface markers like CD11c, CD16, CD44, CD80, CD86 and CD163 was quantified after 24 h of co-incubation of monocytes with AdMPs in the presence or absence of IFN- α .

Analysing CD11c and CD44, we observed an increased expression after phagocytosis of AdMPs (Fig. 4A). However, no further increase was seen when monocytes were incubated with AdMPs together with IFN- α . In contrast to this, expression of CD16, CD80 and CD163 was increased after phagocytosis of AdMPs and the additional stimulation of monocytes by IFN- α caused an even further increase in the expression of these molecules (Fig. 4B). The expression of the co-stimulatory molecule CD86 was increased after stimulation with AdMPs alone and we detected a further significant increase in its expression after the addition of IFN- α (Fig. 4C).

As we had observed that the engulfment of AdMPs was mediated at least in part by SCARF1, we were interested to see whether the blocking of SCARF1 also altered the AdMP-induced upregulation of CD80 and CD86. Thus, we examined the effect of SCARF1-blocking antibody on CD80 and CD86 expression on the surface of the monocytes after 24 h. Upon addition of SCARF1-blocking antibody, we observed a significant reduction in CD86 expression (Fig. 4D) compared to monocytes that had phagocytosed AdMPs without blockage of SCARF1. Similarly, we observed a reduction in CD80 expression (statistically not significant, data not shown) when SCARF1-mediated phagocytosis was blocked. As shown in Fig. 4D, addition of IFN- α to the co-culture of AdMPs and monocytes in the presence of SCARF1-blocking antibody resulted in a partial restoration of CD86 expression on the cell surface.

Synergism of AdMPs and IFN- α is mediated through phosphorylation of STAT1

The major question resulting from our observations is what factors are involved in the upregulation of pro-inflammatory cytokine production and cell surface markers when IFN- α and AdMPs act on monocytes. Two major factors known to be involved in IFN- α signalling are signal transducer and activation of transcription 1 and 2 (STAT1 and STAT2) and their phosphorylated counterparts (Platanias, 2005). We therefore performed protein analysis of STAT1 phosphorylation in monocytes incubated with AdMPs in the presence or absence of IFN- α , as well as of monocytes left untreated and those incubated with IFN- α alone (Fig. 5). Relevant differences could be detected. As expected from the interferon signalling pathway, incubation of monocytes with IFN- α alone resulted in an increase in phosphorylated STAT1 (pSTAT1; normalized to total STAT1 protein content) compared to monocytes that were left untreated. In addition, incubation of monocytes with AdMPs alone resulted in an increase in pSTAT1, but not to the same level as IFN- α by itself. Incubation of monocytes with both AdMPs

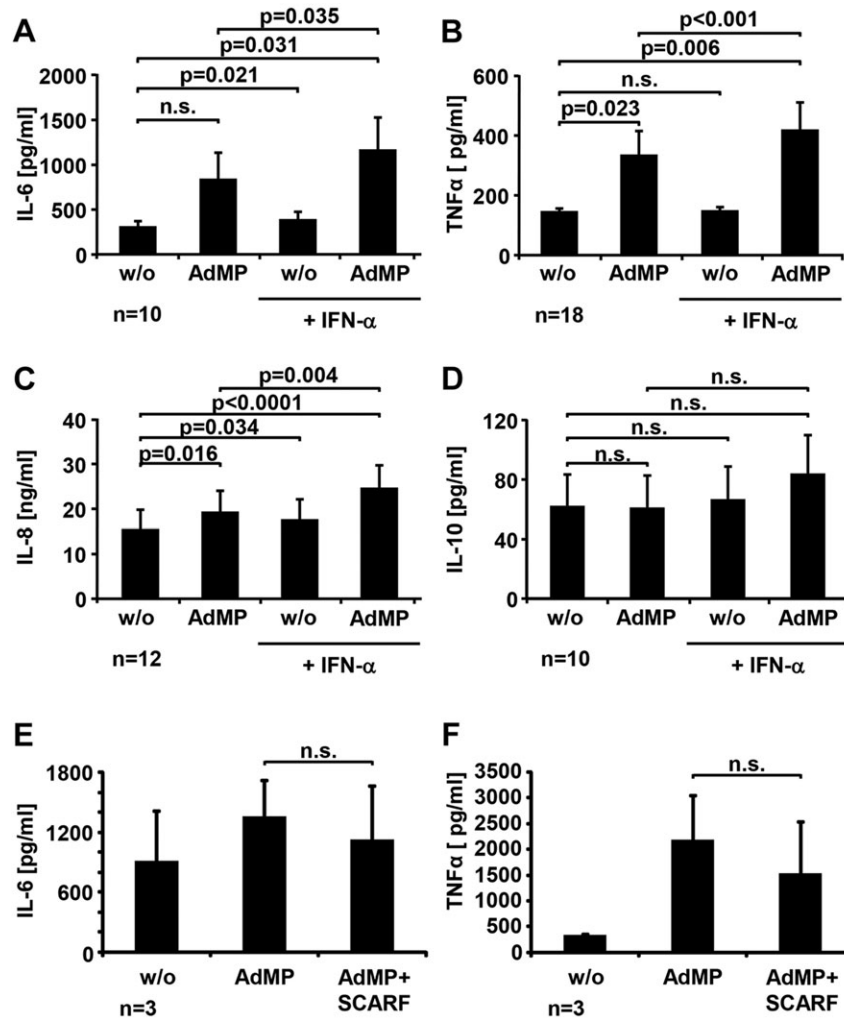


Fig. 3. Cytokines released from monocytes after co-incubation with AdMPs in the presence or absence of IFN- α . (A–D) Monocytes were co-incubated with AdMPs in the presence (AdMP+IFN- α) or absence of IFN- α (AdMP). Cytokines released into the cellular supernatant were quantified after 24 h of incubation. Untreated monocytes (w/o) and cells supplemented with 5 ng/ml IFN- α alone (w/o+IFN- α) served as a control. The graphs show mean+s.e.m. values obtained from at least 10 independent experiments (as indicated in the figure). Statistical significance was calculated by employing a Student's *t*-test, n.s. indicates no significant difference. (A) IL-6. AdMPs caused an increase in IL-6 concentration (+AdMP). AdMP uptake in the presence of IFN- α significantly increased the amount of IL-6 even further (AdMP+IFN- α). (B) TNF- α . Addition of AdMPs (+AdMP) significantly increased TNF- α levels. In the presence of IFN- α (AdMP+IFN- α) this increase was even higher. No increase was seen with IFN- α alone (+IFN- α). (C) IL-8. Stimulation with AdMPs (+AdMP) resulted in significantly higher IL-8 levels. AdMPs in combination with IFN- α (AdMP+IFN- α) caused a highly significant increase in IL-8 concentrations compared to cells untreated. (D) IL-10. No significant change was seen upon stimulation with AdMPs (+AdMP), IFN- α (+IFN- α) or a combination of the two (AdMP+IFN- α). (E,F) Monocytes were also co-incubated with AdMPs in the presence (AdMP+SCARF) and absence of 0.5 μ g/ml SCARF1-blocking antibody (AdMP). Untreated monocytes (w/o) served as a control. The graphs show mean+s.e.m. values obtained from three independent experiments. Statistical significance was calculated employing the Student's *t*-test, n.s. indicates no significant difference. (E) IL-6. Addition of SCARF1-blocking antibody to the co-culture of monocytes with AdMPs (AdMP+SCARF) resulted in a decrease of IL-6 levels detected in their supernatant compared to those without blockage of SCARF1-mediated AdMP uptake (AdMP). (F) TNF- α . Levels of TNF- α detected in the supernatants of co-cultures of monocytes with AdMPs in the presence of SCARF1-blocking antibody (AdMP+SCARF) was lower compared to the concentrations detected when phagocytosis of AdMPs was not inhibited by SCARF1-blocking antibody (AdMP).

and IFN- α , however, resulted in an even higher amount of phosphorylated STAT1 than IFN- α by itself. This increase in phosphorylated STAT1 was significant compared to the level of pSTAT1 detected in samples from untreated monocytes (Fig. 5B).

AdMPs and IFN- α shift macrophage differentiation towards a pro-inflammatory phenotype

As described above, stimulation of monocytes with AdMPs together with IFN- α caused an increase in pro-inflammatory cytokine secretion and an upregulation of co-stimulatory molecules and proteins involved in antigen presentation. Thus, we wanted to investigate the influence of these two factors on the functional

properties of monocyte-derived macrophages. For this purpose, we generated classically activated M1-like macrophages and an alternatively activated less inflammatory type of macrophage (M2-like). Polarisation of the macrophages was controlled by analysis of the cytokine content in the supernatants of untreated cells. As shown in Fig. 6A, M1-like macrophages exhibited a highly pro-inflammatory phenotype, with secretion of high amounts of the pro-inflammatory cytokines IL-6, IL-8, TNF- α and the p40 (IL12B) subunit of IL-12 and IL-23, the latter being pathognomonic for classically activated macrophages. In contrast, there was a generally much lower secretion of pro-inflammatory cytokines by M2-like macrophages, indicating a less inflammatory type of cell (Fig. 6A).

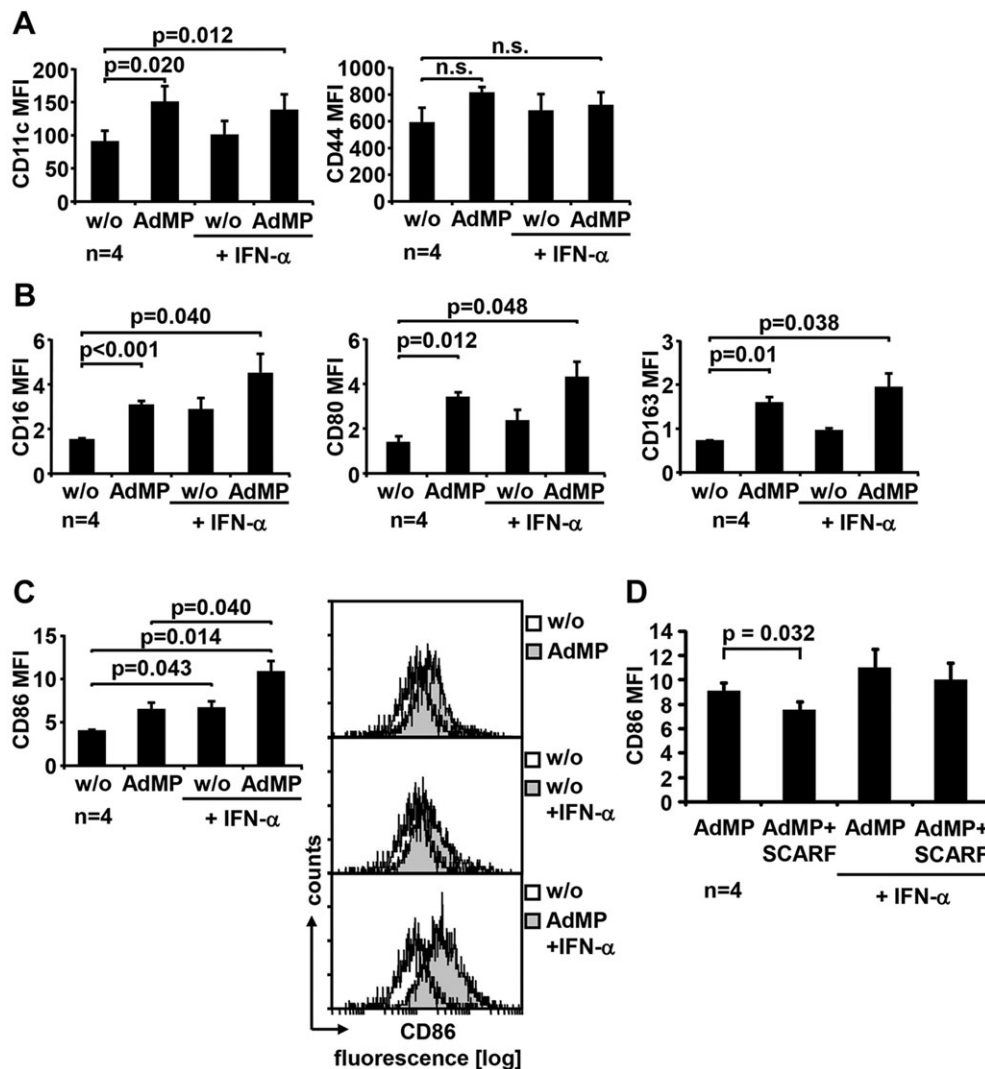


Fig. 4. Regulation of cell surface marker expression on monocytes after stimulation by AdMPs and IFN- α . Monocytes were co-incubated with AdMPs in the presence or absence of IFN- α . After co-incubation for 24 h, the surface expression of CD11c, CD16, CD44, CD80, CD86 and CD163 was quantified by analysing the MFI. In addition, the effect of SCARF1-blocking antibody on CD86 expression was evaluated. (A) CD11c and CD44 surface expression on AdMP-stimulated monocytes was quantified. AdMPs alone caused an increase in CD11c and CD44 surface expression. The additional treatment with IFN- α (+IFN- α) did not cause a further increase. (B) The surface expression of CD16, CD80 and CD163 is shown. Here, AdMPs caused an increase in surface expression (+AdMP), which was further increased by an additional treatment with IFN- α (AdMP+IFN- α). (C) The left graph shows the surface expression of CD86 on AdMP-stimulated monocytes (AdMP). The additional treatment with IFN- α (AdMP+IFN- α) caused a further, significant increase in CD86 expression compared to AdMP stimulation alone (+AdMP). The histograms on the right show original data obtained from one representative experiment. In all experiments, untreated cells (w/o) and cells supplemented with IFN- α alone (+IFN- α) were analysed as a control. (D) Monocytes were incubated with AdMPs in the presence (AdMP+SCARF) and absence of 0.5 μ g/ml SCARF1-blocking antibody (AdMP). Blockage of SCARF1-mediated phagocytosis resulted in a significantly decreased expression of CD86 on the surface of monocytes. Furthermore, the addition of 5 ng/ml IFN- α to the co-culture in which SCARF1 was blocked (AdMP+SCARF+IFN- α) was examined and shown to result in a partial restoration of the CD86 expression. Monocytes incubated with AdMPs in the presence of IFN- α served as a control (AdMP+IFN- α). In the graphs, mean+s.e.m. values obtained from four independent experiments are shown. Statistical significance was calculated employing the Student's *t*-test, n.s. indicates no significant difference.

We then analysed phagocytosis of AdMPs by M1-like and M2-like macrophages. AdMPs were generated from CFSE-labelled activated T-cells and co-incubated together with M1-like and M2-like macrophages. We observed an engulfment of AdMPs by M1-like, as well as, to a lesser extent by M2-like macrophages. Interestingly, in contrast to the results obtained when analysing monocytes (Fig. 1), the additional treatment with IFN- α did not cause a further increase of the engulfment of AdMPs (Fig. 6B).

To analyse further the release of cytokines, co-incubation of macrophages with AdMPs derived from phytohemagglutinin (PHA)-activated lymphocytes was performed in the presence and absence of IFN- α . The cell supernatant was collected after 24 h, and

IL-6, IL-8 and TNF- α secretion was measured. In the supernatants of the classically activated M1-like macrophages, the amount of pro-inflammatory cytokines was already high if cells had been left untreated. Upon stimulation with AdMPs or IFN- α plus AdMPs, a slight increase was detected in the cytokine secretion of TNF- α and IL-8. However, this increase was not statistically significant (Fig. 7A). We observed no changes in IL-6 secretion after stimulation either by AdMPs or by IFN- α in M1-like macrophages.

In contrast to this, we observed that the less inflammatory M2-like macrophages were shifted towards a pro-inflammatory phenotype after stimulation with AdMPs. The generally much lower concentration of pro-inflammatory cytokines in the supernatants of

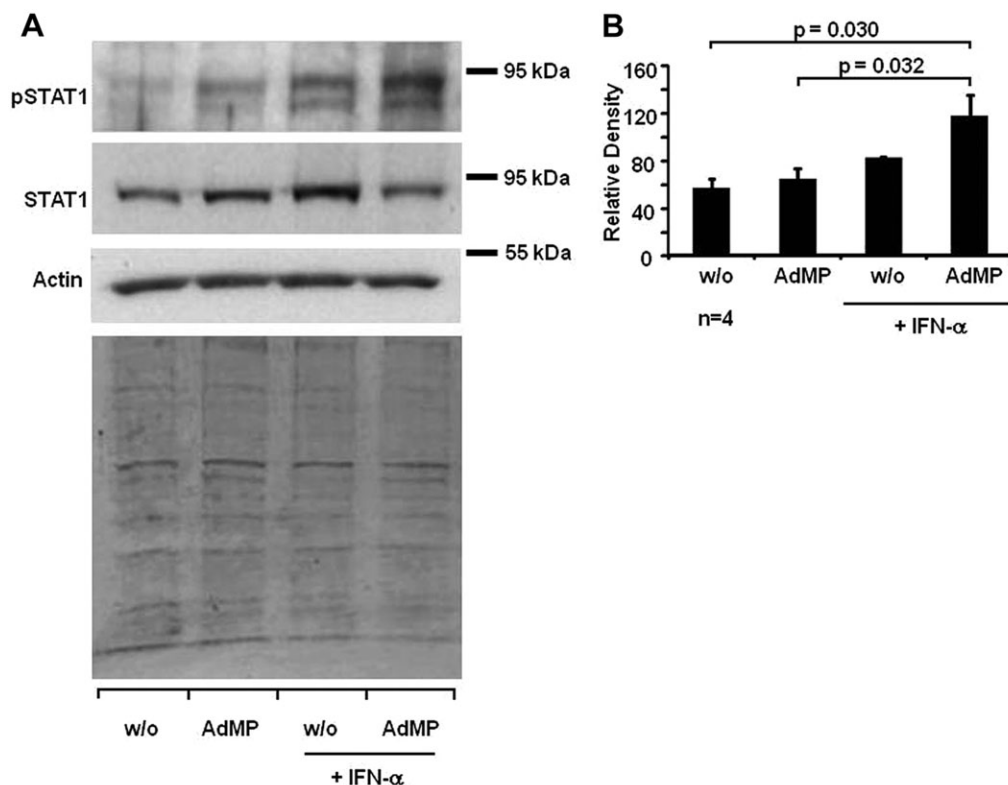


Fig. 5. Protein analysis of monocytes after stimulation with AdMPs and IFN- α . Monocytes were incubated for 24 h under four different conditions. Monocytes alone (w/o) were compared to monocytes incubated with either AdMPs or IFN- α (5 ng/ml) alone as well as to monocytes incubated with both AdMPs and IFN- α (AdMP+IFN- α). Monocytes were then harvested and protein analysis for STAT1 expression and STAT1 phosphorylation was performed. The western blot shows data of 1 representative experiment (Fig. 5A). Data obtained for pSTAT1 and total STAT1 protein are shown in the upper two blots. As a control for the equal loading of protein to each lane an actin western blot was performed and total SDS page is shown. Further, the western blot data obtained from four independent experiments were analyzed by optical densitometry. The data obtained for phosphorylated STAT1 was normalized to total STAT1 protein content (Fig. 5B). Mean values+s.e.m. are shown. Statistical significance was calculated by the Student's *t*-test.

untreated M2-like macrophages was increased twofold when the cells were incubated with AdMPs (Fig. 7B). In addition, similar to the secretion pattern observed in monocytes, the combination of AdMPs and IFN- α induced an even higher secretion of TNF- α and IL-6 (Fig. 7B). Thus, treatment of the less inflammatory M2-like macrophages with AdMPs caused a shift towards a more inflammatory type of cell. This effect was further enhanced if M2-like macrophages were treated with AdMPs in combination with IFN- α .

In addition, analysis of macrophage cell surface marker expression revealed a much higher expression of CD80 and CD86 on M1-like macrophages compared to M2-like macrophages. Consistent with the findings in cytokine production, we observed a highly significant increase in CD80 and CD86 expression on M2-like macrophages compared to cells left untreated upon incubation with AdMPs and IFN- α (data not shown), whilst no significant difference in expression of CD80 and CD86 was seen on M1-like macrophages.

DISCUSSION

In this study, we focused on two major pathogenetic factors of SLE. On the one hand, IFN- α has been considered a major player in SLE. Serum levels of IFN- α have been shown to correlate with disease activity (Becker-Merok et al., 2013; Ytterberg and Schnitzer, 1982). Furthermore, gene expression profiling studies have come up with a number of dysregulated interferon-inducible genes in SLE, the so-called 'interferon gene signature' (Baechler et al., 2003). On the other hand, apoptotic material has been described to accumulate in SLE and is thought to be responsible for the induction of autoimmunity. Only recently have AdMPs been identified as possible regulators in immune responses. In addition, there is evidence to suggest that they can act as auto-adjuvants in the pathogenesis of autoimmune diseases. In this context, it has

been shown that autoantigens accumulate within AdMPs after induction of apoptosis (Schiller et al., 2008). Furthermore, AdMPs have been shown to modulate the function of plasmacytoid dendritic cells (the main producers of IFN- α) as well as myeloid dendritic cells (Fehr et al., 2013; Schiller et al., 2012).

In the present study, we wanted to examine the interaction between pathologically raised levels of IFN- α and circulating AdMPs when they encounter monocytes or monocyte-derived macrophages. Strikingly, a positive correlation between levels of IFN- α and disease activity has been reported (Ytterberg and Schnitzer, 1982), and the SLE disease activity index (SLEDAI) has been found to correlate negatively with levels of circulating microparticles (Sellam et al., 2009). Our finding of an IFN- α -dependent increase in phagocytosis of AdMPs by monocytes (Fig. 1C) might provide an explanation for these findings. According to our results, high levels of IFN- α might stimulate the clearance of AdMPs from the circulation. As we have previously shown, AdMPs themselves are able to increase the production of IFN- α by pDCs (Heyder et al., 2007). These findings, together with the data presented in this study, indicate a possible auto-adjuvant function of the AdMPs in their own removal. Consistent with these findings are results published by Manfredi et al. (1998), who showed that immune complexes formed between autoantigens and autoantibodies, which circulate at high rates in SLE, also promote the uptake of apoptotic cells. Interestingly, we were able to show that the uptake of AdMPs into monocytes partly depends on the scavenger receptor SCARF1. Blockage of SCARF1 by a blocking antibody resulted in a significant reduction in phagocytosis. A similar mechanism has been shown in uptake of apoptotic cells (Ramirez-Ortiz et al., 2013) by dendritic cells. Not only did SCARF1-deficient mice show a despaired removal of apoptotic cells, but also with time Ramirez-Ortiz et al. were able to detect autoantibodies typical for SLE in those SCARF1-deficient mice.

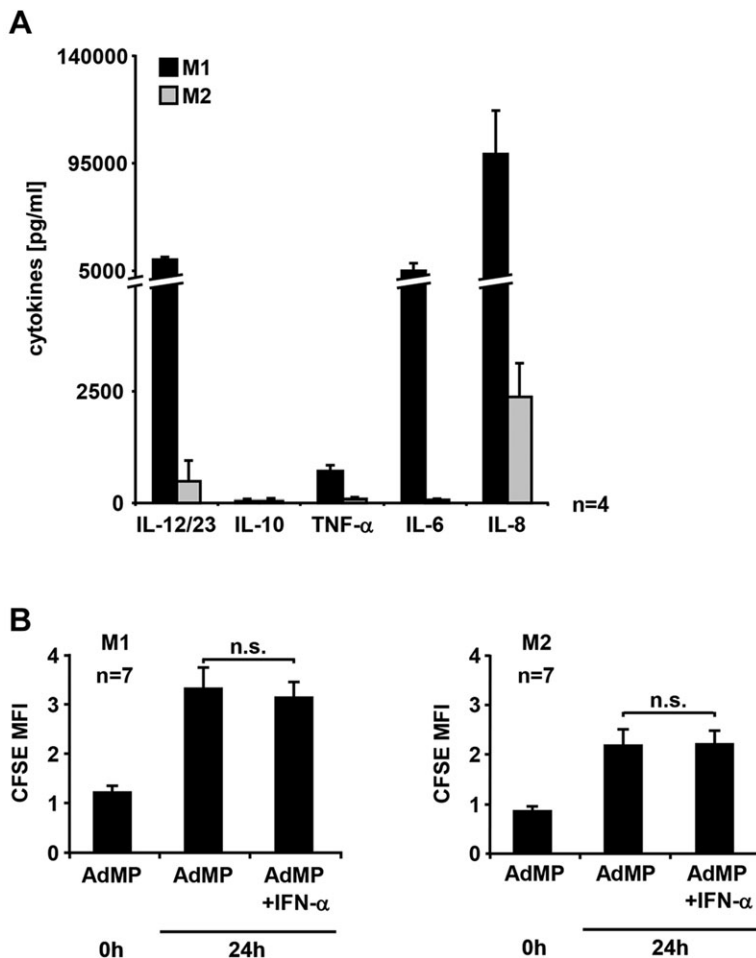


Fig. 6. IFN α does not influence the uptake of AdMPs by M1-like and M2-like macrophages. (A) Cytokine profile of *in-vitro*-generated M1-like and M2-like macrophages. M1-like and M2-like macrophages were generated *in vitro*. Cytokines released into the cellular supernatant were quantified. M1-like macrophages (black bars) released large amounts of pro-inflammatory cytokines [p40 subunit of IL-12 and IL-23 (IL-12/23), TNF- α , IL-6 and IL-8], whereas M2-like macrophages (grey bars) released these cytokines to a much lesser extent. Mean+s.e.m. values obtained from four independent experiments are shown. (B) Engulfment of AdMPs by M1-like and M2-like macrophages. M1-like (left graph) and M2-like (right graph) macrophages were co-incubated with isolated AdMPs (AdMP) in the presence or absence of IFN- α (AdMP+IFN- α). As a negative control macrophages were analysed directly after addition of AdMPs (0 h). After 24 h of incubation engulfment of AdMPs was quantified by means of flow cytometry. The MFI (gating on macrophages) is shown in the graphs. The graphs show mean+s.e.m. values obtained from seven independent experiments. Statistical significance was calculated employing the Student's *t*-test, n.s. indicates no significant difference.

Our data provide further evidence for involvement of SCARF1 in the removal of apoptotic microparticles. However, in our experiments the presence of high levels of IFN- α led to a diminishment of the observed reduction in phagocytosis. In disorders such as SLE this might be of particular relevance as high levels of circulating IFN- α could facilitate accumulation of apoptotic debris.

The role of antibody-facilitated uptake of apoptotic cells and microparticles has also been focus of intensive research within the past years. IgG and also recently IgM have been shown to play a role in uptake of not only pathogens, but also apoptotic material (Litvack et al., 2011; Peng et al., 2005). Importantly, our results show that addition of polyglobulin did not result in a higher amount of AdMP uptake, suggesting that antibody-mediated uptake of AdMPs plays only a minor role in our cellular system.

We have shown that the engulfment of AdMPs, especially in the presence of IFN- α , results in the establishment of a pro-inflammatory environment. Stimulation of monocytes by AdMPs caused an increased secretion of pro-inflammatory cytokines. At the same time, we observed a significant upregulation of the co-stimulatory markers CD80 and CD86, especially in the presence of both AdMPs and IFN- α . IFN- α has been shown to generate potently antigen-presenting cells, as it is able to induce a nondendritic antigen-presenting cell derived from monocytes, termed IFN-Mo (Gerlini et al., 2008). In combination with granulocyte macrophage colony-stimulating factor (GM-CSF, also known as CSF2) and IL-4, the classical differentiation of monocytes into dendritic cells is enhanced (Paquette et al., 1998). In the present

study, the stimulation of monocytes with AdMPs in combination with IFN- α resulted in a significant increase in the expression of co-stimulatory molecules (Fig. 4). Furthermore, AdMPs in combination with IFN- α caused an increased release of the pro-inflammatory cytokines IL-6, IL-8 and TNF- α . These data indicate that AdMPs and IFN- α synergistically support the generation of a pro-inflammatory environment. This hypothesis is strongly supported by our findings that blockage of SCARF1-mediated AdMP uptake decreases the secretion of the pro-inflammatory cytokines IL-6 and TNF- α (Fig. 3E,F), as well as the expression of the co-stimulatory surface markers CD80 and CD86 (Fig. 4D) on the surface of those monocytes, as compared to cells that were not inhibited in their phagocytosis of AdMPs. In diseases that are triggered by the accumulation of apoptotic material (like SLE), then this mechanism might help propagate pathogenic immune reactions.

Recent studies have suggested an involvement of several members of the STAT family in the pathogenesis of SLE (Hedrich et al., 2014; Huang et al., 2011). STAT1 in particular has been associated with the development of, for example, lupus nephritis (Dong et al., 2007) and purpose anaemia (Dominguez-Gutierrez et al., 2014) and also has been shown to be hypomethylated in CD4⁺ T-cells from lupus patients (Coit et al., 2013) as well as having higher expression in peripheral blood mononuclear cells (PBMCs) from SLE patients (Karonitsch et al., 2009). Our results show that the synergism observed between IFN- α and AdMP on inflammation is likely to be mediated by a readily increased phosphorylation and therefore higher transcriptional activity of STAT1.

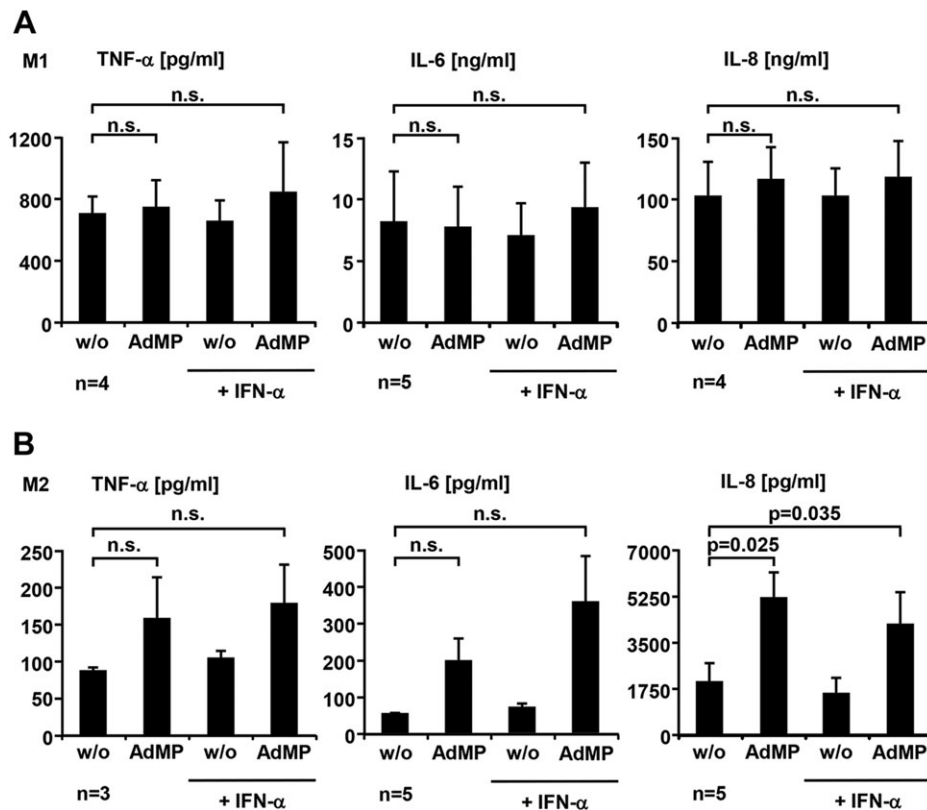


Fig. 7. Cytokines released by M1-like and M2-like macrophages after stimulation with AdMPs and IFN- α . M1-like and M2-like macrophages were stimulated by isolated AdMPs (+AdMP) in the presence (+IFN- α) or absence of IFN- α . Cytokines released into the cellular supernatant were quantified after 24 h of incubation. Untreated macrophages were analysed as a control (w/o). (A) The amount of cytokines released (TNF- α , IL-6 and IL-8) from M1-like macrophages was not significantly changed by either AdMPs or IFN- α . (B) The amount of cytokines released by M2-like macrophages after stimulation by AdMPs, IFN- α or AdMPs plus IFN- α is shown. AdMPs (+AdMP) caused an increased secretion of proinflammatory cytokines (TNF- α , IL-6 and IL-8). The release of TNF- α and IL-6 was further enhanced by an additional stimulation with IFN- α . Mean+s.e.m. values are shown in the graphs. Statistical significance was calculated employing the Student's *t*-test, n.s. indicates no significant difference.

Additional signals, for example during infections, might even potentiate this inflammatory reaction. This could also explain the fact that a disease flare is often seen in patients with SLE after an infection. Thus, in SLE patients, elevated levels of IFN- α together with the accumulation of AdMPs might contribute to the perpetuation or initiation of an inflammatory response, if apoptotic material accumulates in the presence of an elevated IFN- α level.

Interestingly, we observed striking differences comparing the engulfment of AdMPs by monocytes and macrophages (see supplementary material Table S1). The presence of IFN- α did not alter the engulfment of AdMPs by M1-like or M2-like macrophages. However, analysing M2-like macrophages, AdMPs caused a shift towards a pro-inflammatory type of cell. This was further supported in the presence of IFN- α (Fig. 7B).

Taken together, we have shown that AdMPs in combination with IFN- α cause the establishment of an inflammatory situation. The observed effects were selective for AdMPs, which pose a rich source of autoantigens, and do not apply to whole apoptotic or necrotic cells. These findings support our hypothesis that the accumulation of AdMPs might contribute to the auto-inflammatory immune response in patients with an increased rate of apoptosis or a defective clearance of apoptotic cells. As this pro-inflammatory effect was further enhanced by IFN- α , this might be a relevant mechanism in human SLE.

MATERIALS AND METHODS

Cell preparation and culture conditions

After informed written consent had been obtained, 40–80 ml of whole venous blood was taken from SLE patients or NHDs. Patients had to fulfil the revised American College of Rheumatology (ACR) criteria for SLE diagnosis from 1982 in order to be included in the study. Almost all patients were subject to steroidal and/or other immunosuppressant therapy such as Azathioprine, Hydroxychloroquine, Methotrexate or Cyclosporine. For

details of patients, see supplementary material Table S2. As healthy controls, only persons without severe medical conditions and no known autoimmune affliction or family history were allowed to participate. This study was performed with the approval of the local ethics committee for usage of patients' and healthy donors' material for scientific research and was carried out according to the ethics guidelines of our institution and the Declaration of Helsinki.

Isolation of peripheral PBMCs was performed by density gradient centrifugation with lymphocyte separation medium (LSM 1077; PAA, Pasching, Austria). Cells were washed with phosphate-buffered saline (PBS) (PAA, Pasching, Austria) and remaining platelets were removed by a fetal calf serum (FCS) cushion (Invitrogen, Darmstadt, Germany). Control of viability of the cells was performed by Trypan Blue staining.

For isolation of monocytes, magnetic cell sorting using CD14 magnetic beads in magnetic activated cell separation (MACS) columns was performed (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was controlled for by flow cytometry (~94%). Cells were then cultured in RPMI 1640, supplemented with 4 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin and 100 μ g/ml streptomycin (R10, all reagents obtained from PAA, Pasching, Austria). In addition, 10% heat-inactivated FCS and 100 U/ml GM-CSF (Berlex Laboratories, Seattle, USA) were added.

In order to isolate granulocytes, PMN were obtained after differential centrifugation. Erythrolysis was performed twice, followed by thorough washing with PBS. Cell viability was again controlled for with Trypan Blue staining. The cells were then cultured in medium as described above.

Generation of macrophages

Two types of macrophages were generated: pro-inflammatory macrophage (M1-like) and a less inflammatory type of macrophage (M2-like). For this purpose, CD14+ cells were cultured in culture medium supplemented with 20% heat-inactivated FCS. For generation of M1-like cells, 200 U/ml GM-CSF was added, whereas 100 ng/ml macrophage colony-stimulating factor (M-CSF) (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for generation of M2-like macrophages. After 7 days, final polarisation of the macrophages was achieved by adding 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich Chemie, Steinheim, Germany) plus 20 ng/ml interferon- γ

(IFN- γ) (Peprotech, Hamburg, Germany) for M1- and 20 ng/ml IL-4 (Immunotools, Friesoythe, Germany) for M2-polarisation for further 18 h of incubation. Correct differentiation was controlled by analysis of cytokine secretion and flow cytometrical phenotypisation.

Generation of activated T-cells

Generation of activated T-lymphocytes (PHA-lymphoblasts) was performed by culturing isolated PBMCs in culture medium supplemented with 10% heat-inactivated FCS. For activation of these cells, 1 μ g/ml PHA (Sigma-Aldrich Chemie, Steinheim, Germany) and 500 IU/ml IL-2 were added for 5 days. Subsequent expansion of the cells was achieved by supplementation of IL-2 (Chiron, Uxbridge, UK) alone for another 3 days of culture.

Induction of cell death and isolation of AdMPs

Apoptosis of PMN or activated T-lymphocytes was induced by UV-B irradiation. In order to induce necrosis, cells were boiled at 56°C for 30 min.

For isolation of AdMPs, cells were incubated for 16 h after induction of apoptosis. Then, cell supernatant was collected and two centrifugation steps followed in order to remove any remaining cells. Isolation of AdMPs from the supernatant was performed as described previously (Schiller et al., 2008). In short, ultracentrifugation was performed at 100,000 *g* for 40 min after the supernatant had passed through a filter with a pore size of 1.2 μ m (Syringe filter Minisart, single use) (Neolab, Heidelberg, Germany). The pellets, consisting of AdMPs, were then harvested and their concentration was measured by quantifying their DNA content by means of spectrophotometry. On average 80 μ g of dsDNA was obtained after isolation of 100 million PMN cells. Homogeneity of all MMP preparations was controlled in respect to size and granularity by flow cytometry. For all experiments the MMP suspension was adjusted to an optical density at 260 nm (OD_{260nm}) of 0.09 on an Eppendorf Bio Photometer (Eppendorf, Hamburg, Germany).

ELISA

Supernatants of monocytes and macrophages were collected after an incubation period of 24 h with different preys and/or IFN- α . Analysis was performed for IL-6, IL-8, IL-10 and TNF- α . In addition, macrophage polarisation was controlled for by analysis of the p40 subunit of IL-12 and IL-23, IL-6, IL-8 and TNF- α content in the supernatants of untreated polarised macrophages. All ELISA assays were performed using half-area 96-well plates according to the manufacturer's instructions (R&D Systems, Abingdon, UK). An EL808 Ultra Microplate Reader (BIO-TEK Instruments, Winooski, USA) was used for test analysis.

Flow cytometry

Flow cytometric analysis was performed for purity control after magnetic cell sorting of CD14⁺ cells, cell surface marker analysis and evaluation of phagocytosis by monocytes and macrophages using an EPICS XL flow cytometer (Beckman Coulter, Krefeld, Germany).

For analysis of phagocytosis, PMNs or lymphoblasts were stained with CFSE (3 mM for 10 min at 20°C) (Sigma-Aldrich Chemie, Steinheim, Germany) prior to induction of apoptosis or necrosis. After 16 h, isolated AdMPs, or whole apoptotic and necrotic cells, were incubated with phagocytes for 24 h in the presence or absence of 5 ng/ml IFN- α (Miltenyi Biotec, Bergisch Gladbach, Germany). Phagocytosis was measured by determining the MFI gated on the phagocytes in flow cytometry after 24 h. In addition, baseline phagocytosis (0 h) was determined immediately after addition of phagocytic prey to the culture. For experiments with monocytes, PMNs were used as a source of apoptotic and necrotic material. Macrophages were incubated with AdMPs derived from T-lymphoblasts. Cells were harvested by application of accutase and PBS supplemented with EDTA (Merck, Darmstadt, Germany). A dilution series of IFN- α with monocytes engulfing AdMPs were performed with 1, 5, 10 and 50 ng/ml IFN- α .

Analysis of factors involved in uptake of AdMPs was performed by incubation of monocytes with either 0.5 μ g/ml SCARF1-blocking antibody (anti-human SREC-I/SR-F1 antibody; R&D systems, Wiesbaden-Nordenstadt, Germany), 8 μ g/ml human BAI1 antibody (R&D systems, Wiesbaden-Nordenstadt, Germany) or 1 μ g/ml polyglobulin solution (Octagam 10%, octapharma, Lachen, Switzerland) before incubation with

CFSE-labelled AdMPs for 24 h. Phagocytosis was analysed by flow cytometry.

Analysis of surface molecule expression patterns was also performed after the phagocytes had been incubated for 24 h under different conditions (treatment with AdMPs in the presence or absence of IFN- α). Cells were stained by phycoerythrin-labelled antibodies against CD11c, CD14, CD44, CD80, CD86 (all obtained from Beckmann Coulter, Krefeld, Germany), and CD163 (BD Biosciences, Heidelberg, Germany). MFI was determined by flow cytometry.

Confocal microscopy

For confocal microscopy, cells were harvested and transferred into glass-bottomed chamber slides for analysis on a PerkinElmer Ultra-View spinning disc confocal on a Nikon TE2000-E Ti inverted microscope by using a 100 \times oil-immersion lens [excitation at 488, 568 and 647 nm; detection at 650 nm (shown red) and 488 nm (shown green)]. For the analysis of phagocytosis, CFSE-labelled AdMPs (350 μ g/ml DNA) were co-incubated with 1×10^6 phagocytes per ml. Phagocytes were stained with phycoerythrin-labelled anti-CD14 antibody (BD Biosciences, San Jose, CA).

Transmission electron microscopy

AdMPs from T lymphocytes were fixed by adding glutaraldehyde at a final concentration of 2%. After washing, the samples were post-fixed with 2% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide [$K_4Fe(CN)_6$], contrasted in bloc with uranyl acetate, dehydrated with a graded dilution series of ethanol, and embedded into glycid-ether-100-based resin. Ultrathin sections were cut with a Reichert Ultracut S ultramicrotome (Leica Microsystems) and contrasted. Sections were examined with a Zeiss EM 10 CR electron microscope.

Western blot analysis

Monocytes from NHDs were isolated and cultured in a 48-well-plate at a concentration of 1 million per ml in R10. 200 U/ml GM-CSF was added. AdMPs from PMN of the same donor were generated. On day 1 of culture monocytes were either left untreated, incubated with AdMPs at a concentration of 20 μ g/ml either alone or with IFN- α (5 ng/ml), or with IFN- α (5 ng/ml) alone. Monocytes were then harvested after 24 h and centrifuged at 1600 *g* for 5 min. The sample was then resuspended in lysis buffer (RIPA, ccpro, Neustadt, Germany) containing a protease inhibitor cocktail (complete Mini, Boehringer, Mannheim, Germany). Lysis was performed for 20 min on ice. After centrifugation at 13,500 *g*, the supernatant was carefully removed and transferred into a new microfuge tube. Each sample was diluted in loading buffer and subjected to a standard SDS-PAGE. After transfer onto PVDF membranes (Fisher Scientific, Schwerte, Germany), pSTAT1 and total STAT1 were detected by STAT1 or pSTAT1 antibodies (Cell Signaling/New England Biolabs GmbH, Frankfurt am Main, Germany). Equal loading and transfer of protein was verified by reprobing the membrane with a polyclonal anti-actin antibody (Sigma-Aldrich, Missouri, USA).

For densitometry, films were developed and digitally photographed, and densitometric analysis of the bands of interest was performed. Densitometry was performed by analysing optical intensity values at a resolution of 300 dpi. Band intensities of pSTAT1 bands were normalized to the intensity of total STAT1 protein bands by the formula [(pSTAT1/STAT1) \times 100].

Statistical analysis

Statistical analysis was performed using the paired two-tailed Student's *t*-test using Microsoft Excel software. $P < 0.05$ was taken as the level of significance.

Acknowledgements

Confocal microscopy was performed at the Nikon Imaging Center at the University of Heidelberg. We acknowledge Dr U. Engel and Dr C. Ackermann for technical support. We are grateful to Hilmar Bading, Department of Neurobiology and Interdisciplinary Center for Neurosciences, University of Heidelberg, for providing the opportunity to carry out the electron microscopy work in his laboratory. We thank Andrea Hellwig for her excellent technical support.

Competing interests

The authors declare no competing or financial interests.

Author contributions

A.N. was responsible for study conception and design, data acquisition, data analysis and interpretation and manuscript writing. P.H. was responsible for study conception and design and data acquisition. S.K. was responsible for data acquisition. N.B. was responsible for critical manuscript revision. L.-O.T. was responsible for data analysis and interpretation and critical manuscript revision. H.-M.L. was responsible for data analysis and interpretation and critical manuscript revision. M.S. was responsible for study conception and design, data analysis and interpretation and critical manuscript revision. All authors read and approved the final manuscript.

Funding

This work was supported by a research grant from the German Research Foundation [project Lo437/5-3]; the German Ministry of Education and Research (IMPAM) and the Wucherpennig Foundation.

Supplementary material

Supplementary material available online at
<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.162735/-/DC1>

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