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OSCAR-collagen signaling in monocytes plays a pro-inflammatory role and may contribute to the pathogenesis of rheumatoid arthritis

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Abbreviations: Col, collagen type; FDR, false discovery rate; OSCAR, osteoclast associated receptor; RA, rheumatoid arthritis.

Keywords:

OSCAR, collagen, inflammation, autoimmunity, rheumatoid arthritis, monocyte

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Abstract

Osteoclast Associated Receptor (OSCAR) is an activating receptor expressed by human myeloid cells. Collagen type I (ColI) and collagen type II (ColII) serve as ligands for OSCAR. OSCAR-collagen interaction stimulates RANK-dependent osteoclastogenesis. We have recently reported that OSCAR promotes functional maturation of monocyte-derived dendritic cells (moDCs). OSCAR is up-regulated on monocytes from rheumatoid arthritis (RA) patients with active disease, and these monocytes show an increased pro-osteoclastogenic potential. In the current study, we have addressed a functional role for an OSCAR-collagen interaction on monocytes. We show that OSCAR-ColII signaling promoted the survival of monocytes. Moreover, ColII stimulated the release of pro-inflammatory cytokines by monocytes from healthy donors, which could be completely blocked by an anti-OSCAR monoclonal antibody. Mononuclear cells from the synovial fluid of RA patients plated on ColII secreted TNF-α and IL-8 in an OSCAR-dependent manner. Global RNA profiling showed that components of multiple signaling pathways relevant to RA pathogenesis are regulated at the transcriptional level by OSCAR in monocytes. Thus, OSCAR can play a pro-inflammatory role in monocyte-derived cells and may contribute crucially on multiple levels to RA pathogenesis.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by a dysregulated immune response and profound cartilage and bone destruction [1]. Infiltration of the synovium by pro-inflammatory immune cells is the hallmark of RA pathogenesis [2;3]. These cells secrete multiple cytokines and matrix-degrading enzymes causing tissue damage. Monocytes are believed to crucially contribute to the pathophysiology of RA at multiple levels [4]. The circulating monocytes of RA patients have an activated phenotype characterized by production of pro-inflammatory cytokines IL-1β and IL-6 as well as by up-regulation of the cell surface marker, integrin CD11b [5;6]. Monocytes massively infiltrate the progressively inflamed RA synovium and produce large amounts of the master pro-inflammatory cytokine, TNF-α, thus critically contributing to the sustained joint inflammation. In the course of inflammation, monocytes serve as precursors for macrophages, dendritic cells and osteoclasts [4]. In a number of cross-sectional studies, synovial sublining monocyte/macrophage infiltration was shown to correlate with scores of disease activity in RA patients [7]. Therapeutic targeting of monocytes/macrophages has been suggested for treatment of RA [8-10].

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The remarkable plasticity of monocytes is influenced by the RA joint microenvironment, resulting in an altered differentiation, activation and maturation status [11]. Among microenvironmental factors influencing the immune cell activation in RA patients, the extracellular matrix (ECM) has been suggested to be of importance. It has been speculated that in the joints of RA patients collagens become exposed due to profound synovial architectural reorganization, thus enabling interaction with ECM receptors [12]. Fragments of ColII, the major collagen of the articular cartilage [13], have been detected in synovial fluid (SF) of patients with joint diseases [14-16]. Several studies have addressed ECM interaction with immune cells. ColI has been shown to stimulate cytokine secretion by PBMCs [17] and by synovial fluid mononuclear cells from RA patients [18], albeit, the molecular mechanisms of the ECM-mediated cell activation still remain obscure. We have recently shown that ColI and ColII stimulate cytokine secretion and functional maturation of monocyte-derived DCs via the OSCAR signaling pathway [19]. OSCAR has been described as an important player in "osteoimmunology" [20]. OSCAR is associated with the FcRγ [21] and functions as a stimulatory co-receptor in osteoclastogenesis signaling via the nuclear factor of activated T cells c1 (NFATc1) [22]. OSCAR is expressed in human myeloid cells including monocytes, macrophages, dendritic cells and osteoclasts and is capable of modulating their immune response [19;21;23;24]. Expression of OSCAR in human neutrophils was reported previously by Merck et al [21;24], but was not confirmed in the recent study by Barrow et al [25]. The ECM proteins ColI, ColII [12;19] and ColIII [12] serve as naturally occurring ligands of OSCAR. It has been proposed that OSCAR plays a role in skeletal [26] and joint disorders [27;28]. Monocytes isolated from RA patients express high levels of OSCAR and show an elevated activation status [27]. Furthermore, high levels of soluble OSCAR (sOSCAR), which is presumably membrane-shed or a secreted splice form, have been found in plasma,

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synovial fluid and vasculature of RA patients [27-29]. The plasma level of sOSCAR has been reported to positively correlate with bone destruction and cardiovascular risk in patients with RA [29]. In contrast, a reverse correlation of sOSCAR present in the serum of RA patients with the disease activity was reported by the group of Schett [27]. Both studies were done on small patient populations and with different methodologies; thus the prognostic value of sOSCAR and its functions remain unclear.

The function of OSCAR in monocytes was previously addressed with the use of cross-linking mAb [24]. The authors have shown that triggering of OSCAR induces the release of multiple chemokines and promotes survival of the cells. Cross-linking of OSCAR in the presence of TLR ligands, but not alone, potentiated TLR-induced release of pro-inflammatory cytokines [24]. Recently, Barrow and co-authors [25] reported that OSCAR is expressed on the cell surface of inflammatory interstitial lung and blood $CCR2^+$ monocytes from COPD patients and serves as a receptor for Surfactant Protein D (SP-D). The cells exposed to SP-D secreted TNF-α in an OSCAR-dependent manner. In the current study we addressed the OSCAR-mediated response of monocytes in the presence of ColII, probably one of the most relevant OSCAR ligands in the context of articular cartilage damage and synovial inflammation in RA patients, both processes known to be mediated to a major degree by monocyte-derived cells. We show here that peripheral blood monocytes exposed to ColII increased their viability in an OSCAR-dependent manner. OSCAR engagement by ColII induced profound secretion of pro-inflammatory cytokines, most of which turned out to be regulated at the transcriptional level by OSCAR-ColII signaling, either directly or indirectly. Our data suggest that OSCAR-ColII interaction in monocytes may directly contribute to the sustained inflammation and joint damage in the RA patients.

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Results

OSCAR-ColII interaction supports viability of monocytes

We have previously shown that OSCAR-ColII interaction can promote survival of DC under the conditions of growth factor withdrawal [19]. Merck and co-authors have shown that cross-linking of OSCAR promotes survival of monocytes [24]. Here, we tested whether the naturally occurring OSCAR ligand ColII can support monocyte viability. Purified monocytes were cultured on ColII for two days in the absence of the pro-survival factor, M-CSF. Cell viability was analyzed by flow cytometry using Annexin V staining (to detect cells undergoing apoptosis). A majority of the monocytes deprived of M-CSF stained positive for Annexin V on day 2 (Fig.1). All monocytes cultured in the presence of M-CSF (used as positive control) were Annexin V negative (i.e. viable). Similarly, ColII promoted survival of the monocytes, as approximately 80% of the cells stained negative for Annexin V. When OSCAR-ColII signaling was blocked with an anti-OSCAR mAb cell viability was drastically decreased, which was not the case when an isotype control mAb was used.

OSCAR-ColII signaling stimulates secretion of pro-inflammatory mediators

We investigated the effect of OSCAR-ColII interaction on the ability of monocytes to secrete pro-inflammatory mediators. Freshly purified monocytes from six healthy donors were stimulated with ColII for 20 hours and the cell culture supernatants were analyzed for the presence of inflammation markers, such as cytokines, chemokines and acute-phase reactants, using the Multi-Analyte Profiling (MAP) technology platform (service provided by Myriad RBM Inc.). Of the 45 different factors analyzed, 19 inflammation markers were detected

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(shown in Table 1), of which 14 were significantly up-regulated upon ColII exposure as compared with the untreated control, while five showed a trend for up-regulation. Stimulation with ColII triggered production of Alpha-1-Antitrypsin (AAT), Beta-2-Microglobulin (B2M), Fibrinogen, IL-1β, IL-1ra, IL-8, MIP-1α, MIP-1β, Vascular endothelial growth factor (VEGF), RANTES, tissue inhibitor of metalloproteinases (TIMP)-1, TNF-α, TNFR2 and vitamin D binding protein (VDBP) in monocytes, which were down-regulated to the baseline level when OSCAR-ColII interaction was blocked by an anti-OSCAR mAb, but not by the isotype control. Thus, the production of the aforementioned 14 pro-inflammatory mediators by monocytes was dependent on the OSCAR-ColII interaction. The markers, ICAM1, IL-6, IL-10, IL-18 and MCP-1, showed a trend for up-regulation following stimulation with ColII. To confirm the specificity of the effect of native ColII a triple-helical ColII-derived OSCAR-binding peptide (described in [12]) was used to stimulate monocytes. Similar to ColII, the peptide stimulated TNF-α release from the cells, which was fully blocked by an anti-OSCAR mAb (Supporting Information Figure 1).

OSCAR expressing cells are present in the RA joints and respond to stimulation by ColII

The expression of OSCAR was analyzed by flow cytometry on the mononuclear cells from SF and cells from ST of RA patients. Consistent with data on peripheral blood cells from healthy donors [21], OSCAR was expressed on myeloid cells present in SF ($CD14^+$ and $CD86^+$ cells) but not on lymphocytes ($CD3^+$ cells) of RA patients (Fig.2A). In ST of RA patients, OSCAR expression was detected on $CD14^+$, $CD68^+$ and $CD86^+$ cells (Fig.2B), again showing OSCAR is expressed on myeloid cells. Culturing SF mononuclear cells from RA

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patients on ColI has previously been shown to induce secretion of IL-1β, IL-6 and TNF-α [18]; however, the molecular mechanisms controlling such cell activation have not been described so far. Here, RA synovial fluid cells were cultured on plate-bound ColII in the presence or absence of a blocking anti-OSCAR mAb or isotype mAb. After one day, cell culture supernatants were collected and cytokines were analyzed by ELISA. Consistent with our findings in Table 1, the natural ligand of OSCAR, ColII, induced secretion of TNF-α and IL-8 from RA SF cells, which was significantly inhibited by the anti-OSCAR mAb, but not by the isotype control mAb (Fig.3). Our data suggest that OSCAR-expressing myeloid cells are present in the joints of RA patients and can be activated by ColII via the OSCAR signaling pathway.

OSCAR engagement by ColII induces up-regulation of activation markers expressed at the cell surface

We have further analyzed ColII-exposed monocytes derived from healthy donors for the expression of the surface markers, including the activation marker integrin CD11b [5;6], the pro-inflammatory marker Triggering Receptor Expressed on Myeloid cells (TREM-1) [30], and the immune synapse components, HLA-DR, ICAM-1 and CD86 [31]. The monocytes were cultured on ColII for four hours before the expression of surface markers was examined by flow cytometry. We observed that ColII was able to induce up-regulation of the cell surface markers, TREM-1, CD11b, ICAM-1 and CD86, but not HLA-DR (Fig.4). The up-regulation of these surface markers by ColII was abolished when OSCAR-ColII interaction was blocked with an anti-OSCAR mAb, whereas the isotype control mAb had no effect.

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Signaling by OSCAR-ColII regulates gene expression in monocytes

To elucidate the molecular mechanisms of the OSCAR-ColII effects on monocytes, gene expression profiling was performed using a full transcriptome Affymetrix HTHGU133PLUSPM gene chip. Monocytes were cultured for 4 hours on uncoated plates or on ColII-coated plates in the presence or absence of blocking anti-OSCAR mAb or isotype control mAb. The full microarray dataset can be downloaded from the ArrayExpress depository (accession number E-MTAB-3281). ColII treatment significantly changed the expression level of 226 genes (>2 fold change; 5% false discovery rate; Supporting Information Table 1). Pre-treatment of the ColII exposed cells with a blocking anti-OSCAR mAb affected the expression level of 528 genes when compared with pre-treatment with an isotype mAb (Supporting Information Table 2). Of the 226 genes regulated by ColII, 218 were clearly regulated via the OSCAR signaling pathway as the ColII effect was fully abolished by pre-treatment with an anti-OSCAR mAb, but not by the isotype mAb ("true OSCAR-collagen target genes"; Supporting Information Table 3). The OSCAR-ColII regulated genes were clustered into functional categories and pathways using the Ingenuity Pathway Analysis software. The top 20 canonical pathways are listed in Table 2. OSCAR-ColII signaling regulated the expression of genes involved in adhesion/diapedesis, cell activation, immune regulation and a number of genes implicated in various signal transduction pathways implicated in inflammation. OSCAR may directly or indirectly be involved in the transcriptional regulation of pro-inflammatory cytokines, their receptors and components of their signaling pathways. Among those, expression levels of the gene encoding IL-1 (IL1A), its receptor IL1R1 as well as the receptor antagonist IL1RN (coding for the IL-1Ra protein) were up-regulated, which is consistent with our data on the cytokine secretion (Table 1). OSCAR-ColII interaction up-regulated expression levels of the gene

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coding for the key pro-inflammatory cytokine TNF-α as well as the TNF family member TNFSF14 (encoding the LIGHT protein), while the expression level of another TNF-related gene, TNFSF10 (encoding TRAIL) was down-regulated. Genes encoding chemokines (CCL7, CXCL1, -2 and -3, CXCL5, CCL20 and CCL24) were transcriptional targets of the OSCAR-ColII signaling. The most pronounced up-regulation was seen for the gene encoding CCL24, a chemoattractant for eosinophils, neutrophils and macrophages [32]. Other OSCAR-ColII regulated molecules and pathways with relevance to inflammation included components of the NF-κB, TREM-1, Jak2/STAT and TLR signaling pathways as well as ECM modifying enzymes such as MMP7 and MMP19. Genes down-regulated by the OSCAR-ColII signaling included FZD2 (encoding frizzled 2, a receptor for WNT proteins) as well as chemokine receptors, CCR2 and CX3CR1.

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Discussion

The differentiation, polarization and activation of leukocytes are influenced by their interaction with the ECM [17;33;34]; however, it still remains unclear which receptors mediate these effects. We have recently shown that ColI and ColII induce functional maturation and activation of human monocyte-derived DCs through OSCAR signaling [19]. Besides providing structural support, collagens exert an important role in various cellular functions, including proliferation, migration and apoptosis [35]. The structure of ECM is pivotal for defining the cellular response [36]. In RA, joint destruction is a hallmark of the disease. The structural damage leads to an increased turnover of collagens within the joint [37] making collagens accessible for interaction with the infiltrating immune cells, which can thus potentially alter their differentiation and activation. Monocytes, one of the major drivers of synovial inflammation, infiltrate the RA synovial tissue where they encounter collagen. OSCAR-expressing mononuclear cells have been detected in the inflamed RA synovium in contact with the exposed collagens [12;27]. Therefore, we hypothesized that the activation status of monocytes exiting from the circulation into the tissue can be modulated by the exposed collagens in the RA joint microenvironment via OSCAR signaling.

Merck and colleagues have shown that cross-linking of OSCAR promotes survival of monocytes [24]. Here, we examined the functional role of OSCAR stimulation with ColII in monocytes. We showed that peripheral blood monocytes exposed to ColII exhibited increased viability in the absence of growth factors, and that this effect was mediated by the OSCAR pathway. Though the molecular mechanisms of this effect were not addressed in the current manuscript, we speculate, based on the microarray data, that the OSCAR-ColII signaling regulates the expression of genes involved in the control of cell death and survival. Among

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transcriptional targets of OSCAR are genes encoding components of the NF-κB- and ERK/MAPK signaling machinery (both can work in pro-survival pathways, reviewed in [38;39]) as well as members of the death receptor signaling like TNF-Related Apoptosis Inducing Ligand (TNFSF10 or TRAIL) and TNF-α. TNF-α can induce survival of myeloid cells [40-42], while TRAIL triggers programmed cell death and is down-regulated in monocytes stimulated with ColII. TRAIL is linked to the pathogenesis of RA where it has been suggested to inhibit the development of the disease by affecting the viability of the cells in the synovium [43]. We observed an up-regulation of TNF-α at both the gene expression and protein levels, possibly contributing to the increased viability of monocytes in an autocrine/paracrine manner.

We detected secretion of several inflammatory markers when monocytes were exposed to ColII, indicating that ColII also activates monocytes in an OSCAR-dependent manner. In contrast to the data published by Merck *et al*. reporting that OSCAR cross-linking cannot induce secretion of pro-inflammatory cytokines, but only chemokines [24], we detected release of pro-inflammatory cytokines including IL-1β and TNF-α. Our data are in agreement with recently published data by Barrow and colleagues [25], who showed that OSCAR engagement by its novel ligand, SP-D, induces TNF-α release from inflammatory $CCR2^+$ monocytes of COPD patients. The discrepancy between the data from cross-linking studies [24] and the studies by us and Barrow et al [25], where natural OSCAR ligands were used, may possibly be explained by differences in experimental set-ups and/or detection systems used. However, based on the available data we cannot completely rule out the contribution of other receptors necessary for full cell activation by natural OSCAR ligands. IL-1β and TNF-α are key players in RA pathogenesis and are present at high concentrations in the synovial

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fluid of RA patients [44]. They can increase cellular infiltration of the synovium, potentiate osteoclastogenesis and induce MMP production within the RA joint [44-46]. TNF-α was shown to enhance OSCAR expression on monocytes [27], which might amplify the effect via a positive feedback mechanism.

Several chemokines were induced by the OSCAR-ColII interaction. Among the secreted chemokines, IL-8 (recruiting granulocytes), RANTES (recruiting T cells) and MIP-1α/β (chemoattractant for monocytes/macrophages, NK cells and neutrophils) were significantly up-regulated. MIP-1α/β has been shown to stimulate the production of reactive oxygen species and to induce the synthesis of pro-inflammatory cytokines in macrophages and fibroblasts [47-49]. At the level of gene expression, several chemokine genes were up-regulated including CCL24 (recruiting resting T cells and, to a lesser degree, granulocytes), CXCL3 and CXCL5 (both attracting neutrophils and acting on the same receptor, CXCR2) and CCL7 (recruiting monocytes/macrophages). Altogether, these data indicate the potential of OSCAR signaling to contribute to the amplification of the inflammatory response in RA.

The monocyte chemokine receptors, CCR2 (receptor for MCP1) and CX3CR1 (fractalkine receptor) were down-regulated at the gene expression level upon exposure of the cells to ColII in an OSCAR-dependent manner. These receptors, which are critical for the initial recruitment of monocytes, have been reported to be down-regulated upon extravasation of monocytes into the inflamed tissue [50;51] and their sequential differentiation into macrophages [52], which might be essential for cell retention within the inflamed tissue. OSCAR-ColII signaling is likely implicated, at least partly, in these previously described phenomena.

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The expression of several cell surface markers including CD11b, TREM-1, ICAM1 and CD86 was found to be up-regulated in ColII-exposed monocytes in an OSCAR-dependent manner. CD11b is an activation marker on monocytes, whose expression is enhanced in RA patients. It has been proposed that CD11b is involved in monocyte infiltration of the synovium [5]. We have previously shown that OSCAR-collagen signaling is controlling the expression of the major immune synapse components (HLA-DR, CD40, CD80, CD86 and ICAM1) in monocyte-derived DCs [19]. Similar to DCs, monocytes may act as APC, though being less potent. Here, we show that, unlike DCs, ColII-exposed monocytes did not up-regulate HLA-DR, though the expression of the adhesion molecule ICAM1 and the co-stimulatory molecule CD86 was increased upon OSCAR engagement by ColII. Thus, OSCAR may contribute to the monocyte-T cell interaction via stabilization of cell-cell interaction and by enhancing co-stimulatory signaling.

Another surface marker induced by the OSCAR-ColII interaction on the gene expression and protein expression level was TREM-1 and multiple downstream components of the TREM-1 pathway were up-regulated by the OSCAR-ColII signaling in monocytes (Table 2). TREM-1 is a member of the immunoglobulin protein superfamily that activates myeloid cells through the DAP12 signaling. TREM-1 has been shown to amplify TLR and NOD-mediated signaling, where it synergistically enhances cytokine secretion [30;53-56]. TREM-1 has mainly been implicated in bacterial infections [54]. In addition, TREM-1 expression has also been shown to be up-regulated in the RA synovium [57] and blocking TREM-1 signaling in the murine collagen-induced arthritis model significantly improved the disease outcome [58] consistent with TREM-1 being associated with RA pathogenesis.

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We have identified an OSCAR-driven up-regulation of the genes encoding MMP7 and MMP19 in monocytes cultured on ColII. MMP19 has been detected at the surface of activated peripheral blood mononuclear cells [59] and in blood vessels of the inflamed synovium of RA patients [60]. MMP-19 can hydrolyze basement membrane components [61] and constituents of cartilage [62]. This suggests that OSCAR-ColII interaction may potentially enhance extravasation and diapedesis of monocytes during arthritic diseases, leading to an increased invasion of the pannus into the joint, thus contributing to joint destruction.

In RA, the level of OSCAR expression on monocytes has been shown to positively correlate with disease activity and it was suggested that OSCAR functions as a co-stimulatory receptor enhancing osteoclastogenesis from monocyte-derived precursors [27]. In the synovial tissue of RA patients, OSCAR expressing mononuclear cells were localized adjacent to the microvasculature [27;28]. Here, we tested whether cells present in the synovial fluid of RA patients can be activated by ColII via OSCAR. The mononuclear SF cells stimulated with ColII secreted TNF-α and IL-8, which was abrogated by blocking the OSCAR-ColII interaction with an anti-OSCAR antagonistic mAb. Our results support previously published findings on the activation of mononuclear cells from RA patients cultured on collagen [18] and identify OSCAR as being the receptor mediating this effect. Thus, OSCAR is functional in mononuclear cells present in the SF of RA patients and these cells, via the OSCAR-ColII interaction, could contribute to RA pathogenesis by secreting pro-inflammatory cytokines and chemokines. Collagen fragments have been identified in the synovial fluid of RA patients [14;15;63;64]. Though we did not test whether they can serve as functional OSCAR ligands,

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we speculate that they can contribute to activation of the myeloid cells present in the synovial fluid.

Similar to its function in DCs [19], the primary function of OSCAR in monocytes seems to be pro-inflammatory. Our findings indicate the existence of a novel pathway regulating the activity of monocytes exposed to ColII upon tissue remodeling as found e.g. within joints of RA patients. Our data, with both blood monocytes and mononuclear SF cells from RA patients, suggest that the OSCAR-ColII interaction could be important in perpetuating the course of the disease in RA patients. Given the importance of monocytes/macrophages for cartilage damage and synovial inflammation targeting of OSCAR-ColII signaling in these cells may represent a novel therapeutic approach for the treatment of RA.

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Materials and methods

Antibodies and ECM proteins

The following monoclonal antibodies (mAb) were used for flow cytometry analysis: Alexa Fluor 647-conjugated anti-OSCAR mAb (Novo Nordisk A/S), APC-conjugated anti-CD14 (BD Pharmigen), FITC-conjugated anti-CD14 mAb (eBioscience), FITC-conjugated anti-CD68 mAb (eBioscience), PerCP-Cy5.5–conjugated anti-CD3 mAb (eBioscience), FITC-conjugated anti-HLA-DR mAb (BD Pharmigen), PE-Cy5-conjugated anti-CD11b mAb (BD Pharmigen), APC-conjugated anti-TREM1 mAb (BioSite), PE-Cy5-conjugated anti-ICAM1/CD54 mAb (BD Pharmigen), PE-Cy5-conjugated anti-CD86 mAb (eBioscience) in experiments with RA patients material and PE-conjugated anti-CD86 mAb (IOTest) in experiments with samples from healthy donors. The isotype control mAb used were anti-TNP conjugated with Alexa Fluor 647 (Novo Nordisk A/S), IgG1κ-FITC (eBioscience), IgG2a-PerCP-Cy5.5 (eBioscience), IgG2B-PE (R&D systems), IgG1κ-APC (BD Bioscience), IgG2bκ-FITC (BD Pharmigen), IgG1κ-PE-Cy5 (BD Bioscience) and IgG1κ-PE (BD Bioscience).

For functional assays, the OSCAR blocking humanized IgG1.1 mAb (described in Ref. [19]) was used. As an isotype control, an anti-TNP humanized IgG1.1 was used. IgG1.1 antibodies harbor a human Fc fragment with five point mutations, which prevents binding of the Fc receptors [65].

ColII purified from human cartilage was purchased from Millipore.

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The level of endotoxin in all protein solutions used for functional studies was measured using the turbidimetric kinetic LAL assay (Charles River Laboratories). All proteins were essentially endotoxin-free (below detection limit, <0.01 EU/ml).

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte-enriched buffy coats by Ficoll-Plague PLUS (GE Healthcare) density centrifugation. Buffy coats from healthy individuals were obtained anonymously from the Blood Bank, The State University Hospital (Rigshospitalet), Copenhagen, Denmark. All donors gave informed consent according to the protocol approved by The Ethics Committee for Copenhagen, Denmark, for research use (ethical approval number H-D-2008-113). Red blood cells (RBC) were removed using a lysis buffer (eBioscience). Monocytes were purified from PBMCs using CD14 Microbeads human positive selection kit (MACS; Miltenyi Biotec) according to manufactures' protocol using an AutoMACS Pro (Miltenyi Biotec). The purity of the $CD14^{+}$ preparations was assessed by flow cytometry.

Synovial fluid (SF) from anonymous RA patients from Peking University People's Hospital (PKUPH), Beijing, China, was obtained by needle aspiration. All donors gave informed consent according to the protocol approved by the Ethics Committee of PKUPH for research use (approval #20110513-LZ-PKUPH). Synovial fluid cells were isolated by centrifugation at 300 xg for 10 min.

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Synovial tissue (ST) from RA patients was obtained during knee replacement surgery at PKUPH. The tissue was placed in a buffer (1g tissue per 3 ml buffer) containing 4 mg/ml Collagenase A (Roche) and 0.1 mg/ml of DNase I (Roche) in RPMI 1640 medium and then homogenized using a MACS dissociator. The samples were incubated on a MACS mix tube rotator at 37°C for 30 min. The reaction was stopped by adding an ice-cold growth medium (RPMI 1640 with 10% FCS). The cell suspension was filtered using a 70 μm strainer and centrifuged at 300 xg for 10 min. The cells were re-suspended in a growth medium and processed for flow cytometry analysis.

Activation of cells by plate-bound collagen

For monocytes of healthy donors, 24-well plates were coated with ColII in PBS at ~3μg/cm^2 overnight at 4°C. The plate was washed three times in PBS before cells were added to the wells. 1x10^6 cells were seeded per well in growth medium (RPMI1640 with GlutaMAX) (Invitrogen, Life Technologies) supplemented with 10% heat-inactivated FCS and 1% penicillin-streptomycin (GIBCO, Life Technologies), pre-incubated 15-30 min with anti-OSCAR or isotype control mAb (1μg/ml) as indicated. The cells were cultured on ColII for various time points as indicated. In some of the experiments, 100ng/ml LPS (Sigma) was included as control.

RA SF cells (5x10^5 cells /well) were cultured on high binding 96-well cell culture plates coated with ColII as described above.

Analysis of cytokine secretion

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Supernatants of monocyte cultures were collected after 24 hours of ColII exposure, frozen at -80°C and shipped to Myriad RBM (Austin, TX) for a 45-Biomarker Multi-Analyte Profiling.

Supernatants from SF cells cultured for 24 hours on ColII were collected and analyzed using ELISA. The TNF-α ELISA kit was from eBioscience and the IL-8 ELISA kit was from BD Bioscience.

Monocyte viability

Monocytes were washed twice and re-plated in fresh growth medium without cytokines at $5x10^5$cells/ml. The monocytes were left untreated or plated onto ColII-coated plates for 2 days in the absence or presence of anti-OSCAR mAb (1µg/ml) or isotype control (1µg/ml). As a positive control, M-CSF (10ng/ml) was added. At day two, the cells were washed in PBS and stained with Annexin V-FITC (BD-Pharmigen) for 15 min at room temperature followed by flow cytometry analysis using a FACSFortessa™ (BD Bioscience).

Analysis of cell surface markers by flow cytometry

Expression of cell surface markers was assessed by flow cytometry. In short, cells were washed twice in PBS with 2% FCS and then stained for 30 min at 37°C in PBS containing 2% FCS in the presence of 1% human serum albumin (HSA) to block unspecific binding. Cells were rinsed twice in PBS with 2% FCS prior to flow cytometry analysis. Data acquisition was done using a FACSFortessa™ (BD Bioscience). Data analysis was performed using Kaluza software version 1.2 (Beckman coulter).

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Microarray analysis

Total RNA from monocytes of six donors was obtained four hours after stimulating the cells with ColII in the absence or presence of anti-OSCAR mAb or isotype control mAb. RNA was extracted using TRIzol (Invitrogen, Life Technologies) followed by purification with an RNeasy MinElute Cleanup Kit (Qiagen). The RNA integrity was evaluated on an Agilent 2100 Bioanalyser using Agilent RNA 6000 Nano Kit chips (Agilent Technologies), with RNA Integrity Number (RIN) scores of 9.2 or above. Microarray experiments and data normalization were performed as described in [19]. A paired two-group comparison was done with Benjamini-Hochberg correction for multiple testing applying a false discovery rate (FDR) of 5% and a biological threshold of more than 2-fold regulation. The effect of ColII treatment and the effect of pre-treatment with an anti-OSCAR inhibitory mAb versus an isotype control mAb were evaluated using Qlucore Omics Explorer software. Overlapping genes, i.e. genes regulated by ColII and reversed in the presence of anti-OSCAR mAB were subjected to Gene Ontology (GO) analysis for biological processes using Ingenuity Pathway Analysis (IPA). The dataset and technical information compliant with MIAME can be found at the EMBL-EBI ArrayExpress depository, accession number E-MTAB-3281 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3281/).

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 software. The results are shown as mean + standard deviation (SD) unless otherwise indicated. Differences between mean values were analyzed using a One-way ANOVA test with Dunnett’s correction of multiple comparisons to

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a single control. Differences were considered significant when P-values were below 0.05 ($P < 0.05$). The asterisks indicate: *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

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Conflict of Interest Disclosure

The authors declare no commercial or financial conflict of interest.

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Figure legends

Figure 1. OSCAR-ColII interaction supports survival of monocytes. Freshly purified monocytes from healthy donors were cultured on either untreated or ColII-coated plates. To the ColII coated wells, OSCAR blocking mAb or control mAb (1µg/ml) were added as indicated. M-CSF (10ng/ml) was used as positive control. As a negative control monocytes were left in M-CSF- free medium. After 2 days, cell viability was assessed using Annexin V

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staining. For flow cytometry analysis, (A) single cells were gated in (left) a SSC-H vs SSC-W scatter, then (right) debris was gated out in a SSC vs FSC scatter. This gating strategy was used for all donors. (B) Representative histograms are shown for the cells gated in (A). Numbers in the corners show the percentage of cells negative for Annexin V (viable). Data are shown for one representative donor out of four analyzed. (C) Percentage of alive cells (Annexin V^{neg}) is quantified from histograms as shown in (B) for four independent donors. Data are shown as mean of four donors ± SD, each donor represented by a dot. Significance was calculated in relation to untreated by one-way ANOVA followed by a Dunnett's post test.

Figure 2. OSCAR-expressing cells are present in synovial fluid and synovial tissue of RA patients. (A) For flow cytometry analysis, single cells were gated in a SSC-H vs SSC-W scatter (left, gate A), only cells negative from for the dead cell marker Near-IR was used in the analysis (middle, gate B), then cell debris was gated out using FSC vs. SSC (left, gate C). (B and C) This population (gate C) was further analyzed using multicolor staining for the indicated markers. OSCAR expression was analyzed on (B) synovial fluid cells which were positive for $CD14^+$, $CD86^+$ or $CD3^+$; and (C) on the cells from synovial tissue which were positive for $CD14^+$, $CD86^+$ and $CD68^+$. Blue histograms represent OSCAR staining; red histograms – isotype control staining. Data are shown from one representative patient out of ten analyzed.

Figure 3. ColII induces inflammatory cytokine secretion from RA synovial fluid cells. (A and B) Synovial fluid cells were plated on ColII for 20-24 hours in the absence or presence of

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anti-OSCAR mAb or isotype control mAb (10μg/ml) in quadruplicate wells. The supernatants were analyzed for (A) TNF-α and (B) IL-8 expression using ELISA. Data are shown as mean ± SD (n=4) and are from one representative patient out of five analyzed. Significance was calculated in relation to ColII* by t test.

Figure 4. Cell surface markers expressed on the monocytes cultured on ColII. (A and B) $4x10^6$ monocytes ($CD14^+$) were pre-treated with either anti-OSCAR mAb or isotype control mAb (1μg/ml) for 30 min, transferred onto ColII-coated plates and incubated for four hours. As a positive control, LPS (100ng/ml) was used. Cells were analyzed by multicolor flow cytometry for surface expression of the indicated markers. (A) Single cells were gated in a SSC-H vs SSC-W scatter (left, gate A), followed by gating out cell debris in a SSC vs FSC scatter (right, gate B). (B) The expression levels of HLA-DR, TREM1, CD11b, CD86 and ICAM1 are presented as MFI values (median fluorescence intensity). Data analysis was performed using Kaluza software. Data pooled from four donors are shown as mean ± SD (n=4). p-values are determined by one-way ANOVA with Dunnett's post test.

Table 1. Inflammatory mediators secreted by monocytes exposed to ColII

	monocytes	ColII-monocytes	ColII-monocytes + anti-OSCAR	ColII-monocytes + isotype
AAT (ng/ml)	79.0±4.8	92.0±4.9*	67.0±3.0*	96.0±7.1**
B2M (μg/ml)	0.12±0.013	0.16±0.014***	0.10±0.011	0.16±0.017***
Fribrinogen (ng/ml)	6.9±0.94	10±2.1*	7.2±1.0	10±2.3*
ICAM-1 (ng/ml)	0.49±0.018	0.67±0.10*	0.53±0.027	0.59±0.048
IL-1b (pg/ml)	6.3±2.7	15±5.3*	2.9±0.55	14±4.4

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IL-1ra (pg/ml)	147±32	797±213**	90±14	807±198**
IL-6 (pg/ml)	19±11	51±24	17±6.3	41±18
IL-8 (ng/ml)	0.39±0.18	1.4±0.57*	0.21±0.048	1.2±0.39
IL-10 (pg/ml)	3.4±0.82	4.8±1.2	3.3±0.58	4.9±1.3
IL-18 (pg/ml)	6.3±1.2	7.8±1.3	5.4±1.0	7.1±1.3
MIP-1a (pg/ml)	24±8.3	58±11***	14±3.7	60±9.6***
MIP-1b (pg/ml))	153±50	331±52***	108±28	334±54***
MCP-1 (pg/ml)	17±5.9	29±9.1	15±3.2	30±8.4
RANTES (pg/ml)	3.2±0.39	4.2±0.59***	2.6±0.32*	3.9±0.42*
TIMP-1 (ng/ml)	22±6.0	54±14**	13±2.5	55±15**
TNF-a ((pg/ml)	22±7.4	89±23***	11±2.0	85±19**
TNFR2 (ng/ml)	2.1±0.23	3.2±0.26***	1.4±0.13**	3.2±0.2***
VEGF (pg/ml)	307±83	419±83*	212±31	367±61
VDBP (ng/ml)	1.2±0.13	1.4±0.16**	1.1±0.11	1.4±0.14*
Ferritin (ng/ml)	101±16	84±11	92±16*	88±10
IL-1a (pg/ml)	0.63±0.11	0.66±0.11	0.43±0.036	0.7±0.083

$CD14^+$ monocytes were either pre-incubated with 1µg/ml anti-OSCAR mAb or an isotype control for 30 min before plating onto ColII-coated plates for 20 hours. Supernatant was collected, frozen and shipped to Myriad RBM in Austin, TX for a 45-biomarker Multi-Analyte profiling. AAT: Alpha-1-Antitrypsin; B2M: Beta-2-Microglobulin; MIP, Macrophage Inflammatory Protein; TNFR2: Tumor Necrosis Factor Receptor 2; TIMP-1: Tissue Inhibitor of Metalloproteinases 1; VEGF, Vascular Endothelial Growth Factor; VDBP: Vitamin D-Binding Protein. $p<0.05$=*; $p<0.01$=**; $p<0.001$=*** indicate significant difference from untreated.

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Ingenuity Canonical Pathways	Molecules	-Log (*p*-value)
Granulocyte Adhesion and Diapedesis	MMP7,IL1A,CCL20,CCL24,IL1R1,CXCL5,SDC4,CXCL3,IL1RN,SDC2,CXCL1,CXCL2,TNF,MMP19,CCL7	9,72E+00
Agranulocyte Adhesion and Diapedesis	MMP7,IL1A,CCL20,CCL24,IL1R1,CXCL5,SDC4,CXCL3,IL1RN,CXCL1,CXCL2,TNF,MMP19,CCL7	8,38E+00
Atherosclerosis Signaling	IL1A,IL1RN,LPL,APOC1,CCR2,F3,TNF,TNFSF14,TNFRSF12A	5,52E+00
Role of IL-17A in Psoriasis	CXCL3,CCL20,CXCL1,CXCL5	5,27E+00
TREM1 Signaling	CXCL3,NLRP12,TLR8,TLR7,JAK2,TNF,CCL7	5,09E+00
LXR/RXR Activation	IL1A,IL1RN,LPL,SERPINF1,APOC1,IL1R1,TNF,CCL7	4,50E+00
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	IL1A,F2RL1,RRAS,IL1RN,TLR8,TLR7,CEBPD,IL1R1,JAK2,TNF,FZD2	3,77E+00
Role of IL-17A in Arthritis	CXCL3,CCL20,CXCL1,CXCL5,CCL7	3,71E+00
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	IL1A,SPP1,IL1RN,TLR8,TLR7,TNF	3,70E+00
Acute Phase Response Signaling	IL1A,RRAS,IL1RN,SERPINF1,IL1R1,JAK2,SERPINE1,TNF	3,62E+00
NF-κB Signaling	IL1A,RRAS,IL1RN,TLR8,TLR7,MAP3K8,IL1R1,TNF	3,58E+00
PPAR Signaling	PPARG,IL1A,RRAS,IL1RN,IL1R1,TNF	3,55E+00
FXR/RXR Activation	PPARG,IL1A,IL1RN,LPL,SERPINF1,APOC1,TNF	3,44E+00
IL-17A Signaling in Airway Cells	CXCL3,CCL20,CXCL1,CXCL5,JAK2	3,38E+00
Colorectal Cancer Metastasis Signaling	MMP7,RRAS,TLR8,TLR7,RHOU,JAK2,TNF,FZD2,MMP19	3,28E+00
Cholecystokinin/Gastrin-mediated Signaling	IL1A,RRAS,IL1RN,CREM,RHOU,TNF	3,27E+00
Toll-like Receptor Signaling	IL1A,IL1RN,TLR8,TLR7,TNF	3,15E+00
Role of Tissue Factor in Cancer	F2RL1,RRAS,CXCL1,JAK2,F3,EIF4E	3,14E+00

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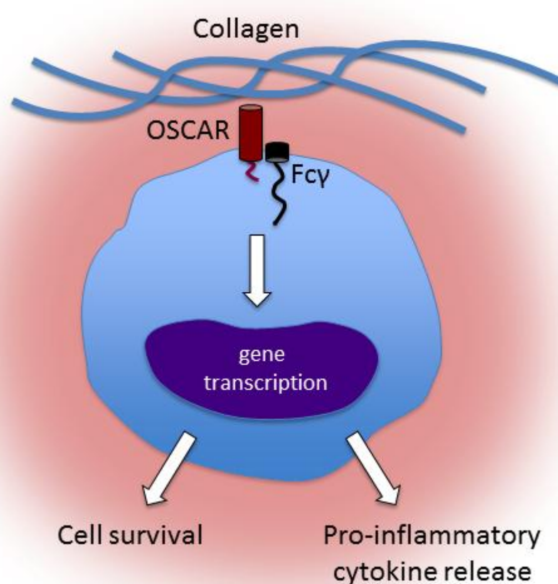
IL-6 Signaling	IL1A,RRAS,IL1RN,IL1R1,JAK2,TNF	3,06E+00
HMGB1 Signaling	IL1A,RRAS,RHOU,IL1R1,SERPINE1,TNF	2,99E+00

Monocytes were cultured on ColII for 4 hours in the presence or absence of anti-OSCAR mAb (1µg/ml) or isotype control (1µg/ml). Total cellular RNA was isolated and genome-wide mRNA expression analysis was done using Affymetrix GeneChip microarray. The table shows canonical pathways for the OSCAR-ColII regulated genes at a false discovery rate of 5% and a biological criterion of minimum two-fold change. A complete list of all genes regulated by OSCAR-ColII signaling is provided in Supplemental Table SIC. Samples from six independent donors were analyzed.

Table 2. Top 20 canonical pathways regulated by the OSCAR-ColII signaling



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