CD56\(^+\) Monocytes Have a Dysregulated Cytokine Response to LPS and Accumulate in Rheumatoid Arthritis and Immunosenescence

Dissertation

zur Erlangung des akademischen Grades Dr. med. an der Medizinischen Fakultät der Universität Leipzig

eingereicht von: Marco Lothar Krasselt


angefertigt am / in: 3. Februar 2014, Leipzig
Klinik für Gastroenterologie und Rheumatologie, Sektion Rheumatologie, Universitätsklinikum Leipzig

Betreuer: Prof. Dr. med. Ulf Wagner,
Dr. rer. nat. Manuela Rossol

Beschluss über die Verleihung des Doktorgrades vom: 18.11.2014
Meiner Mutter.
In ewiger Liebe.
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I. Bibliographic Summary

Krasselt, Marco Lothar

CD56+ Monocytes Have a Dysregulated Cytokine Response to LPS and Accumulate in Rheumatoid Arthritis and Immunosenescence

Universität Leipzig, Dissertation

38 S., 150 Lit., 3 Abb., 1 Tab., 5 Anlagen

Monocytes are no longer regarded as a homogenous cell population but can be divided, both phenotypically and functionally, into different subsets. In rheumatoid arthritis, the subpopulation of CD14bright/CD16+ monocytes is expanded and prone towards generation of Th17 cells. CD56+ monocytes represent a different subpopulation, which is also expanded in conditions associated with autoimmunity like inflammatory bowel diseases. The aim of the study was the quantification and functional characterization of the CD56+ monocyte subset in rheumatoid arthritis (RA).

The work at hand shows that the frequency of CD56+ monocytes is also expanded in RA; moreover, this subpopulation seems to increase with age in healthy controls. This age association is completely lost in patients suffering from RA.

Further functional investigations could demonstrate a dysregulated cytokine response to lipopolysaccharide (LPS) with an increased production of pro-inflammatory cytokines like TNFα as well as an increased spontaneous reactive oxygen intermediate (ROI) production. A longitudinal treatment study using Etanercept as an established TNFα-blocking agent revealed a decrease of the frequency of that cell population under therapy. This decrease was more pronounced in patients with a good treatment response as judged by the reduction of the disease activity score (DAS) 28.

Summing up those results, the CD56+ monocyte subset might be involved in immunosenescence as well as in the pathogenesis of RA.
II. Introduction

Rheumatoid Arthritis (RA) is an inflammatory, autoimmune disease which has a systemic character: being the most common form of inflammatory arthritis, it is not only affecting the human body's joints, but can also lead to a variety of extra-articular manifestations. Though patients are typically suffering from symmetrical joint pain and swelling, symptoms can also include systemic disease manifestations like fatigue and chronic disease's anemia, but also severe problems, e.g. serositis, vasculitis or even interstitial lung disease.

Rheumatoid Arthritis is a common disease with an incidence of approx. 41 new cases per 100,000 people a year [1] and a prevalence of 0.5-1 % [2] in the general population. Incidence appears to be rising with age and peaks among people aged around 70 with rates up to 89 per 100,000. The lifetime risk of developing RA was estimated 3.6 % for women and 1.7 % for men, respectively [3].

The clinical presentation of RA has changed during the last two decades. Crippling arthritis is seen less often, while an early diagnosis due to improved diagnostic tools has become much more common during the last years. This is not only due to the more specific anti-citrullinated proteins/peptides antibodies (ACPA) used to detect the disease, but also due to the recently changed ACR/EULAR criteria of RA allowing diagnosing the disease in an early stage. These improvements are accompanied by the early induction of a sequential anti-inflammatory therapy with a greater variety of potent drugs. It is important to mention the TNFα inhibitors here, the development of which was a huge step forward in treating RA successfully.

Despite all those clinical improvements, deciphering the pathogenesis of Rheumatoid Arthritis is still challenging and further research is clearly required. On the one hand, there are the underlying mechanisms; on the other hand there is a disturbed panel of effector cytokines. It is very important to distinguish between those two points: Influencing the levels of inflammatory cytokines such as TNFα due to (receptor-)blocking agents is one approved clinical way to treat the disease successfully. Why these levels are elevated is a question still not satisfactorily answered yet. Similarly, why TNF inhibitors help some patients while they totally fail in others remains a mystery. A lot of research work today is concentrated on the effector mechanisms, finding new targets or new agents to known targets.

II. 1 Genetics of Rheumatoid Arthritis

The pathogenesis of RA seems to have multiple aspects. Beneath well-known environmental risk factors for developing an RA there are genetic alterations increasing the susceptibility for the disease, especially for the seropositive variant. First hints for the heritability of RA were found due to epidemiologic studies, followed by the comparison of homozygototic and dizygotic twins, where a concordance for RA of 15.4 % for monozygotic but only 3.6 % for
dizygotic twins was shown [4,5]. Overall, genetic factors seem to be responsible for about 60% of RA’s distribution within a population, regarding to quantitative genetic studies [6].

II.1.1 Shared Epitope

When it comes to genetics and RA, it is important to have a closer look on the HLA (Human Leukocyte Antigen)-DRB1 locus and the “shared epitope”. Studies showed HLA-DRB1 being responsible for about one third of the genetic contribution towards RA [7]. The term “shared epitope” is used for a set of HLA-DRB1 alleles, all of which are coding for four amino acids in the 3rd hypervariable region of the DRβ chain belonging to the Major Histocompatibility Complex (MHC) class II which form the actual antigen binding site [8]. Depending on the individual HLA-DRB1 shared epitope susceptibility alleles, the risk of developing RA differs: the DRB1*0401 allele goes along with a relative risk (RR) of about 3, while DRB1*0101 is proposed to have a RR of 1.5. The alleles seem to be very stable within a population, most commonly found throughout Europe are the alleles *0101, *0401 and *0404 (f > 5%) while *0405 and *0901 are the most common ones in Asia. Despite these findings, the shared epitope is associated with RA in different populations, regardless of which alleles are predominantly found [9]. Furthermore, more recent works could show that distinct shared epitopes-positive alleles do not only increase the risk of developing an RA, but are also connected to the disease’s severity in terms of radiologic joint destruction (HLA-DRB1 *0401/*0401, *0401/*0404, *0404/*0404 and *0401/x) and developing an accompanying rheumatoid vasculitis (HLA-DRB1 *0401/*0401, *0401/*0404 and *0101/*0401) [10,11].

As illustrated by these findings, this association is strongly dose-dependent, usually being higher in homozygous or compound heterozygous individuals.

II.1.2 Other Genetic Causes

Other risk genes seem to be less important with a lower RR and, moreover, ethnicity dependent. The PTPN22 gene polymorphism, for example, has been shown to be associated with RA in Western countries, including Europe and the US [12-14]. Studies estimated a frequency of about 10% within the population for Northern America [13,15,16]. In contrary, in Asians, the polymorphism was not even found at all [16]. The actual polymorphism is a single nucleotide polymorphism (SNP) and leads to a gain of function of the tyrosine phosphatase the PTPN22 gene is coding for. This enzyme is involved in the termination of T cell receptor (TCR) signaling, thereby establishing a negative feedback regulation, which is more active in individuals carrying this polymorphism. Taking the mechanism of the coded protein and its enhancement into consideration, one would not primarily think of its involvement in an autoimmune disease, and particularly not in peripheral T cells. It has therefore been proposed that the polymorphism especially affects the thymic T cells, resulting in a compromised negative selection of autoreactive cells [17,18]. The described mechanism could consequently lead to an autoimmune disease and is not exclusive for RA; further studies also found an association of the PTPN22 polymorphism with other autoimmune diseases, namely Graves’ disease, type 1 diabetes mellitus, vitiligo, systemic lupus erythematoses and juvenile idiopathic arthritis [19].
Today, there is a total of 31 RA risk loci known, including STAT4, CD40, CTLA4 and CCR6, just to name a few [20]. Based on our current knowledge, they seem to contribute little to the RR of actually developing the disease, and therefore to the genetic predisposition. The major players here are the SE and PRPN22; they contribute for about 50 % of the genetic disease susceptibility [21].

II.2 Antibodies

In autoimmune diseases, it is hypothesised, that the body’s own immune system is reacting against the body’s very own tissues; in terms of RA, the targets appears to be the synovial membrane, and probably some extra-articular tissues and structures. Unfortunately, RA does not seem to be a typical autoimmune disease. Autoantibodies used for diagnosing RA are Rheumatoid factors (RF), as well as anti-citrullinated proteins/peptides antibodies (ACPA). Surprisingly, both of them are directed against antigens widely distributed also outside the joint. RF are autoantibodies against the Fc fragment of Immunoglobulin G (IgG) and have been known for almost 90 years. Their specificity for RA is low (approx. 79 %), they also occur in other diseases or inflammatory conditions, e.g. Sjögren’s Syndrome or Hepatitis C [22,23]. The more specific ACPA (specificity approx. 95 %) [22,24] are directed against proteins in which an amino acid residue has been converted from arginine to citrullin by deamination, catalysed by the peptidylarginine deiminase (PADI). This posttranslational alteration leads to a different three-dimensional structure of the affected protein and takes place in many tissues all over the body [25]. The most important “citrullinated” proteins in this context are matrix proteins such as vimentin, fibrinogen, keratin and fillagrin. Altered vimentin is even found in the rheumatoid pannus inside the joint [26,27].

Since it has such an important role in creating antigens und subsequently ACPA, it was an almost imperative assumption to look for an association between the PADI and RA. Interestingly, a polymorphism within PADI4 seems to be associated with RA in the Asian population [28-30]. No such association could be demonstrated for Caucasians though, emphasizing the presumed differences of RA’s pathogenesis depending on ethnicity (see also PTPN22 in II.1.2) [31-33].

The role of autoantibodies in the pathogenesis of RA is not fully understood yet. Several studies show that ACPA can be detected very early, even a long time before a clinical manifestation of the disease [34]. ACPA are superior to RF not only in detecting the disease, but also as prognostic indicators of disease activity and joint destruction. Both, ACPA and RF, indicate a poorer radiologic outcome and a higher disease activity independently [35,36]. Of RF negative patients, approx. 20 % are positive for ACPA, while approx. 15-20 % of RA patients only test positive for RF without having ACPA. About one third of all RA patients are negative for both autoantibodies. The highest sensitivity and specificity is therefore reached when testing for both, ACPA and RF with a positive predictive value of almost 100 % [37]. Thus, autoantibodies are used to detect RA and, moreover, to predict the severity of the disease. They seem to be pathognomonic, which is especially true for ACPA, which are
directed against altered matrix molecules as described above. There is also a link to the HLA alleles and their distinct SE: recent studies came to the conclusion, that HLA alleles are rather associated with RF and anti-citrullinated peptide antibody production, than with RA itself [36,38,39].

Despite all those findings, there is still the riddle of the clinically identical, but serologically RF and ACPA negative RA. The existence of this “seronegative” disease variant leads to the assumption, that even the most specific autoantibody is either not necessarily involved in the pathogenesis of the disease itself, or that RA is not a homogenous disease entity.

### II.3 Environmental Factors

Smoking is thought to be the most important and best-examined external risk factor. Depending on the smoking duration and the daily dose, a RR of up to 3.0 was found [40]. Both, tobacco dose and smoking years seem to increase the risk dramatically. Interestingly, again a strong association with a positive status for ACPA was found [40,41]. Moreover, smoking seems to increase the risk for developing seropositive RA in a multiplicative manner, especially in the presence of other (“internal”) risk factors, such as HLA SE or PTPN22 [42]. One proposed explanation is an increased peptide deamination/citrullination in smokers in the presence of HLA SE. However, results from different authors remain conflicting, with some being unable to reproduce the HLA SE/smoking association [41,43,44].

### II.4 Adaptive Immunity

Major players of the adaptive immunity are the T cells. First of all, they can be divided into helper (CD4+) and cytotoxic (CD8+) T cells. Both of them are found in the synovial membrane, where T cells are the greatest cell population, accounting for up to 50% of all cells [45]. That is one of the reasons, why T cells have been thought to be involved in RA pathogenesis throughout the last 3 decades. Another one is the fact, that experimentally induced arthritis could be transferred by using T cells alone, especially CD4+ ones [46-48].

The CD4+ T helper cells themselves can be further divided both functionally and phenotypically into four different subsets: Th1, Th2, Th17 (effector T cells) and Tregs (regulatory T cells). In terms of RA, the Th1 subset and the Tregs, as well as the more recently discovered Th17 subset, are probably of greatest importance.

#### II.4.1 Th1 Cells

The Th1 cells are characterized by a distinct cytokine pattern and mechanism of action. Their signature cytokine is IFNγ, separating them from the Th2 subset especially producing IL-4 and IL-13. For a long time, RA was thought to be a Th1 disease, arising from the rather proinflammatory phenotype of that T cell-subset. The results in studies of experimental arthritis and IFNγ have been conflicting though, questioning the role of Th1 cells in RA [49], especially with regards to the more recently discovered Th17 subset.
II.4.2 Th17 Cells

The characterization of the Th17 subset was a huge step forward in understanding autoimmune diseases, and describes a cell population named after their predominantly produced cytokine (IL-17A), which in itself is able to induce inflammation, angiogenesis and cartilage/bone breakdown [50,51]. Th17 cells are recognized as a distinct subset, differentiating from naïve precursors within an environment containing IL-6, IL-23 and TGFβ [51,52]. Studies could show that they are associated with RA, finding increased IL-17 levels within both, blood and synovial membrane of the patient. Furthermore, there seems to be a correlation between RA-induced joint damage and those cytokine levels [53,54]. Just recently, our group was able to find a link between the adaptive and the innate immunity right here: the Th17 differentiation is (also) being induced by a distinct monocyte subset (CD14brghtCD16+) in culture. This subset is also occurring in increased frequencies within the peripheral blood of RA patients, and correlates with the ex vivo measured Th17 cell amount [55].

II.4.3 Tregs

Tregs are “Regulatory” T cells, which are responsible for suppression of effector T cells like Th1, Th2 and Th17; they are therefore thought to “regulate” the adaptive T cell-response and also to prevent autoimmunity by establishing self-tolerance [56]. Interestingly, several studies could show them to be present in the rheumatoid synovium in increased numbers [57]. Although they are supposed to suppress T cell activity there, they seem to be unable to do so. This notion is supported by the finding, that Tregs in RA patients might have a decreased potential to downregulate TNFα and Interferon-γ production by T cells and monocytes [58,59]. Moreover, TNFα as a main player and pharmacological target in the treatment of RA is found abundantly within patient’s joints and seems to suppress the Tregs’ function itself by decreasing FoxP3 expression. The importance of this mechanism is underlined by the observation, that anti-TNFα therapy using Infliximab leads to an increased amount of Tregs with an increased FoxP3 expression, restoring the cytokine-suppressive Treg function [59].

II.4.4 B Cells

B cells are the second large population of lymphocytes in the rheumatoid synovium. Their interaction with T cells is required for an adequate immune response in healthy human beings due to antigen presentation (=activation) and signal amplification by positive feedback stimulation. This two-directional interaction and the finding of RA-specific autoantibodies implicate their involvement in the complicated pathogenesis of RA. Moreover, germinal centre-like structures, which are a histological hallmark of the disease, can only be found in the synovial membrane of RA patients in which dividing B cells are also present [60]. Besides this finding, B cells are able to produce antibodies even without T cell “help”: the ligation of both, the B cell receptor (BCR) and the Toll-like receptor (TLR) 9 are sufficient for B cell activation and antibody production. This mechanism was shown to lead to RF
production in experimental arthritis [61].

B cells producing RF seem to be able to get help from non-autoreactive T cells (i.e. T cells not responsive to IgG/IgM) by presenting foreign antigens using the RF-specific BCR [62]. Physiologically, at this point there ought to be a failsafe mechanism, leading to a reduced life span of autoreactive B cells and preventing class switching. In short, it is thought that B cells specific for a soluble antigen should receive a negative signal due to the missing complexes, leading to apoptosis. The soluble RF, on the contrary, is able to self-polymerize within the follicle centre, forming immune complexes and therefore acquiring C3d, subsequently resulting in a positive feedback-signal to parent B cells due to extensive activation of the specific Complement receptor (CR) 2 [63]. Summing up those findings, immune complexes can be a positive signal, leading to “self-perpetuating” B cells being involved in the pathogenesis of RA [64]. Reducing those B cell clones is one principle of today’s RA therapy by using the chimeric IgG1 CD20-antibody Rituximab.

II.5 Innate Immunity

Monocytes are an important subset of the human peripheral white blood cells and - besides the polymorphonuclear neutrophils (PMN) - one of the innate’s immune system main actors. They are responsible for phagocytosing and destroying infectious agents and pathogens. While they are less effective against bacteria than PMNs, they are actually able to present phagocytosed and processed antigens to other immune cells like T cells and belong therefore to the so-called Antigen Presenting Cells (APCs). Taking this into consideration, monocytes are basically inflammatory immune cells in the first line of defense, and provide – in their role as APCs – a link to the adaptive immune system. Besides these important functions they can also cause severe damage in the tissue due to both, cytokine production (IL-1β, IL-6 and TNFα) leading to T cell activation/-attraction on the one hand and different enzymatic systems (collagenases, enzymes like NADPH-oxidase generating Reactive Oxygen Intermediates [ROI]) on the other hand [65].

Their role in the pathogenesis of RA is yet not fully understood, but according to the literature, they seem to be especially important after differentiation into macrophages. In this form they are able to migrate to the synovial membrane, where they are found in increased frequencies and are considered to take part in the typical tissue damage in patients suffering from RA after getting activated by cytokines, T cells or receptors like the TLR. Radiologic articular destruction seems to be correlated with the amount of tissue infiltration by macrophages [66,67].

Moreover, most inflammatory cytokines, which are important in RA and which decrease in response to conventional therapy using anti-inflammatory drugs as well as directly acting anti-cytokine therapies are mainly produced by macrophages. This is especially true for TNFα, making macrophages/monocytes the major mediator of inflammation, even if T cells might trigger the initial disease process [65,68,69]. Anti-cytokine therapy is therefore also an anti-monocyte therapy. This assumption is emphasized by the recent findings about reverse
signaling through the transmembrane TNF: as our group was able to show, Infliximab-lgation of tmTNF on monocytes leads to inhibition of the constitutive NF-κB activation, suppresses spontaneous IL-1β production and induces apoptosis within monocytes in RA patients but not in healthy controls [70].

Peripheral monocytes can be further divided into three populations by using their surface markers CD16 (Fcγ receptor) and CD14 (TLR 4) [71,72]. A missing expression of CD16 and a high level of CD14 (CD14<sup>bright</sup>/CD16<sup>−</sup>) characterize the major one (about 85-90 %), followed by CD14<sup>+</sup>/CD16<sup>−</sup> cells (8 %) and the smallest population, CD14<sup>bright</sup>/CD16<sup>+</sup> (4-5 %). This last subpopulation seems to be connected to RA as described in the Th17 section of this thesis. It is already known that the monocyte compartment is disturbed in patients with RA [55,73,74].

More recently, a separate and less well-characterized monocyte subpopulation has been described which is characterized by the expression of the surface marker CD56 [75]. CD56<sup>+</sup> monocytes are found in low frequencies in the peripheral blood of healthy individuals [75,76], patients with Down syndrome [77] and patients with chronic myelomonocytic leukemia [78]. This monocyte subpopulation is expanded in Crohn’s disease [76], produces typical monocyte cytokines and is a more efficient antigen-presenting cell population with regard to the induction of a T-cell alloresponse [75].

The surface molecule CD56 is an isotype of the Neural Cell Adhesion Molecule (NCAM) [79] and usually a typical natural killer (NK) cell marker [80,81], but is also found on some T cells [82,83]. However, it has initially been described on neurons where it is thought to be important in cell adhesion and to enhance the cell-to-cell contact [84-87]. Such an adhesive function has also been suggested for NK cells where it is thought to be involved in effector-target cell-interaction by homophilic bindings between the NKC and its target [88,89]. The function of this new monocyte subpopulation is not yet revealed, but it is suggested to have regulatory tasks in cytotoxicity and might promote Th1-type γδ T-cell responses [90].

II.6 Immunosenescence

The incidence rates of many diseases increase with advancing age, e.g. cancer, severe infections or autoimmune diseases like RA. It is believed that this could be explained by the age-associated alterations of the immune system, termed Immunosenescence [91-93]. The investigation of this phenomenon has been one of the main fields of immunologic research in the last years.

Particularly well examined, especially in terms of RA, is the adaptive immune system with its main players, the T cells. The results obtained so far for the innate system are conflicting, arising from investigations on blood cells in different states of development (i.e. monocyte vs. macrophage) and often from animal samples: lots of the studies concentrate on rodents [94-97]. Taking this into consideration, it is not surprising that, for example, the cytokine secretion of cells from elderly was found to be increased both in human [98-100] and in murine cells [101,102]. This conflict is complicated even further by the fact that many of
those results are obtained from in vitro experiments, while only few in vivo studies were performed. Similar conflicts were found for chemotactic and phagocytotic capacity of monocytes as well, with some studies detecting no age-related effects at all [103-105].

However, widely accepted and approved is a condition called inflame-aging, describing a chronic pro-inflammatory status in the elderly [106,107] probably characterized by a progressive increase of cytokines in serum, especially IL-6 with measurements up to ten fold compared to younger subjects [108-110]. Taking all the described findings into consideration, the assumption of an increased cytokine production in elderly seems to be established most firmly and conclusively.

When thinking of inflammation, reactive oxygen intermediates (ROI) must be mentioned. One of the first and most accepted findings about aging in general was the assumption of free radicals being involved in the aging process more than half a century ago [111]. Depending on their stage, monocytes are able to produce large amounts of ROIs. There is also consensus about a connection between the pathogenesis of RA and immunosenescence, especially of the adaptive immune system [112,113]. RA is probably not caused by a fully competent immune system with autoreactive cells that have not been eliminated by tolerance mechanisms. Rather, it appears to be caused by a prematurely senescent immune system with different age-related alterations like the reduction of the absolute amount of T cells and their diversity, loss of CD28 expression and age-inappropriate telomere shortening [113-115]. These changes are probably leading to autoreactive T cells independent of the pivotal CD28 co-stimulation. Besides these findings, other investigations could also show that age affects the Th1 and Th2-differentiation of CD4+ cells in mice negatively, interestingly without affecting Th17-differentiation. Moreover, the Th17 cells from elderly people seem to produce more IL-17 [116,117]. More recent investigations on rodents brought rather conflicting results with an increased proportion of Th17 cells (naïve and memory cells) in aged mice [118]. On the other hand though, a current study in humans shows an increased differentiation of IL-17 effector cells from naïve CD4+ lymphocytes in the elderly with a decreased frequency of IL-17-producing cells within the memory T cell compartment [119].

Very recently, other investigators even found a reciprocal relationship between Th17 cells and Tregs throughout a human's life with an imbalance towards Th17 when getting older [120]. Summing up these results, they clearly provide support for the assumption of Th17 cells being involved in immunosenescence and probably even in the pathogenesis of RA.

II.7 Considerations for the Study Presented and the Aims Pursued

As shown on the previous pages, the pathogenesis of RA is complicated and only partly understood to date. Circulating antibodies play a pathogenic role, as do different players of the adaptive and innate immune system. Epidemiology shows us that RA is a disease of older patient, similar to different kinds of cancer, arteriosclerosis and some severe infections. Already this fact alone implies the described changes of the immune system termed immunosenescence in the pathogenesis of RA as an autoimmune disease. Further
implications came from investigations like the description of the Th17 subset with the age-dependent changes of that population, their possible link to a monocyte subset, the severely altered T cell department and the “inflame-aging” condition with increased cytokine secretion mainly produced by mononuclear cells. It appears that both arms of the human immune system are altered and probably involved in RA.

After reading the work of Grip et al., showing a connection between a monocyte subpopulation and another inflammatory disease responsive to TNFα blocking agents (Crohn's), the idea of measuring the frequency of CD56+ monocytes as cells of the innate immunity in RA patients and healthy controls was born. In the initial analyses, the correlation of the CD14bright/CD56+ cell frequencies in healthy control with their age was readily detectable, which encouraged us to further investigate this subpopulation, especially in view of the lack of an age-related correlation in the overall patients group. We hypothesized, that a connection of those findings with immunosenescence might exist, which prompted us to characterize those cells further. The possible link was moreover emphasized by the fact that young RA patients around their thirties seem to have more CD56+ monocytes than age-matched healthy donors. Both groups, RA patients and healthy controls, were therefore expanded and functional investigations were performed to investigate the function of this monocyte subset.

The next step was a small longitudinal therapy study using Etanercept, a chimeric fusion protein consisting of the Fc fragment of IgG1 and the soluble human TNF receptor 2 (TNFR2), capable of binding and “inactivating” TNFα. Aim of the study was to investigate a potential influence of the TNF-blocking therapy on the amount of CD56+ monocytes, in correlation with disease symptoms in patients suffering from RA. The patients for this study were recruited from the outpatient clinic within our Rheumatology unit. The results of the investigations mentioned above were published and are further discussed in the enclosed paper.
II.8 References

III. Published Article and Results

The following article was published in *Arthritis Research & Therapy* in October, 2013.
Impact Factor: 4.302
CD56+ monocytes have a dysregulated cytokine response to lipopolysaccharide and accumulate in rheumatoid arthritis and immunosenescence

Marco Krasselt, Christoph Baerwald, Ulf Wagner and Manuela Rossol

Abstract

Introduction: Peripheral blood monocytes are no longer regarded as a homogeneous cell population, but can be differentiated both phenotypically and functionally into various subpopulations. In rheumatoid arthritis, the subpopulation of CD14\textsuperscript{bright}/CD16+ monocyte is expanded and prone towards generation of Th17 cells. CD56+ monocytes represent a different subpopulation, which is also expanded in conditions associated with autoimmunity like inflammatory bowel diseases. The aim of the study was the quantification and functional characterization of the CD56+ monocyte subset in rheumatoid arthritis (RA).

Methods: Frequencies of peripheral blood monocyte subpopulations were analyzed by flow cytometry in 86 healthy controls and 75 RA patients. In 16 patients, anti-tumor necrosis factor (TNF) therapy was initiated, and the CD56+ monocyte frequency was monitored longitudinally. Lipopolysaccharide (LPS)-induced cytokine production of CD56+ and CD56– monocytes was determined by intracellular staining or cytokine secretion assays.

Results: In healthy individuals, 8.6% ± 0.6 of the monocytes co-expressed CD56, with the majority of CD56+ monocytes being CD14\textsuperscript{bright} (7.9% ± 0.5), while only a minor population was CD14\textsuperscript{dim} (0.7% ± 0.1). We found a strong positive correlation between an individual’s age and the frequency of CD56+ monocytes. Upon stimulation with LPS, CD56+ monocytes became more frequently positive for TNF, IL-10 and IL-23 than CD56– monocytes. In addition, CD56+ monocytes spontaneously produced more reactive oxygen intermediates than CD56– monocytes. In RA patients, the frequency of CD56+ monocytes was significantly higher than in healthy controls (12.2% ± 0.9 vs. 7.9% ± 0.5, \(p = 0.0002\)). Treatment of the patients with an anti-TNF blocking agent significantly reduced CD56+ monocyte frequencies (baseline 12.4% vs. 24 weeks treatment 8.0%, \(P = 0.0429\)), and the magnitude of this decrease was found to correlate with the change in disease activity under the therapy.

Conclusion: The CD14\textsuperscript{bright}/CD56+ monocyte subset is expanded in aging individuals as well as in patients with RA. The pro-inflammatory production of cytokines and reactive oxygen species as well as the elimination of those cells in patients with a good response towards TNF inhibiting agents indicates a possible contribution of those monocytes in the inflammatory response in RA.
**Introduction**

Peripheral blood monocytes are not a homogeneous cell population, but represent different subpopulations with distinct functions and cell surface markers. Three major subpopulations can be distinguished by the expression of the cell surface markers CD14 and CD16, classical CD14\(^{bright}\)CD16\(^{-}\) monocytes, nonclassical CD14\(^{dim}\)CD16\(^{+}\) monocytes and intermediate CD14\(^{bright}\)CD16\(^{+}\) monocytes [1]. More recently, a separate and less well-characterized monocyte subpopulation has been described which is characterized by the expression of the neural cell adhesion molecule CD56 [2]. CD56\(^{+}\) monocytes are found in low frequencies in the peripheral blood of healthy individuals [2,3], patients with Down syndrome [4] and patients with chronic myelomonocytic leukemia [5]. This monocyte subpopulation is expanded in Crohn’s disease [3], produces typical monocyte cytokines [2] and is a more efficient antigen-presenting cell population with regard to the induction of a T-cell alloresponse [2].

It is already known that the monocyte compartment is disturbed in patients with rheumatoid arthritis (RA). We and others have observed an increase in the frequency of CD16-expressing monocytes [6-8]. To date, no studies evaluating the presence of CD56\(^{+}\) monocytes have been performed in RA patients.

Herein we report an increased frequency of CD56\(^{+}\) monocytes in patients with RA compared to healthy controls. The occurrence of CD56\(^{+}\) monocytes in the peripheral blood is strongly age-dependent in healthy controls, but this association is lost in RA patients. CD56\(^{+}\) monocytes produce more tumor necrosis factor (TNF), interleukin 10 (IL-10) and IL-23 than CD56\(^{-}\) monocytes, and anti-TNF therapy normalizes the frequency of CD56\(^{+}\) monocytes in RA patients.

**Methods**

**Human participants**

Seventy-five patients with RA were included in the study. The diagnosis of RA was based on the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria for RA [9]. Sixteen patients required therapy with a TNF-blocking agent because of their uncontrolled disease, and therefore etanercept treatment was initiated while concomitant disease-modifying antirheumatic drug therapy was continued. The dynamics of the CD56\(^{+}\) monocyte population were monitored before the initiation of therapy and during the following 24 weeks. The characteristics of the study populations are shown in Table 1.

Eighty-six control subjects were recruited among healthy blood donors (median age 53.5 years, range 22 to 72 years; 33 males and 53 females). The ethics committee of the University of Leipzig approved all experiments with human materials, and informed consent was obtained from all participants.

**Cell isolation and culture**

Human peripheral blood mononuclear cells (PBMCs) were isolated as described previously [10]. Cells were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at a concentration of 1 × 10\(^{6}\)/ml.

**Flow cytometry**

To identify monocyte subsets in the peripheral blood, PBMCs were stained with anti-CD56-allophycocyanin (AF12-7H3), anti-CD14-fluorescein isothiocyanate (TÜK4) and anti-CD16-phycocerythrin (VEP13). Antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

<table>
<thead>
<tr>
<th>Table 1 Characteristics of the rheumatoid arthritis patient cohorts</th>
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<tbody>
<tr>
<td><strong>Characteristics</strong></td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Median age, years (range)</td>
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<tr>
<td>Female/male (n)</td>
</tr>
<tr>
<td>Median disease duration, years (range)</td>
</tr>
<tr>
<td>RF-positive (%)</td>
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<tr>
<td>Anti-CCP-positive (%)</td>
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<tr>
<td>Median CRP, mg/dl (range)</td>
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<tr>
<td>DMARDs (n)</td>
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<tr>
<td>Methotrexate</td>
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<td>Azathioprine</td>
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<td>Leflunomide</td>
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<tr>
<td>Anti-TNF</td>
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<td>Anti-TNF + MTX</td>
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<tr>
<td>Abatacept</td>
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<tr>
<td>Hydroxychloroquine + MTX</td>
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<td>Cyclosporin A</td>
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<td>Cyclosporin A + MTX</td>
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<tr>
<td>Tocilizumab</td>
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<tr>
<td>Rituximab</td>
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<tr>
<td>Without</td>
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</table>

**CCP**, cyclic citrullinated peptide; **CRP**, C-reactive protein; **DMARDs**, disease-modifying antirheumatic drugs; **MTX**, methotrexate; **RA**, rheumatoid arthritis; **RF**, rheumatoid factor; **TNF**, tumor necrosis factor.
Measurement of cytokine production
PBMCs were stimulated for four hours (to measure TNF production) or sixteen hours (to measure IL-10, IL-23 and IL-1β production) with 100 ng/ml ultrapure lipopolysaccharide (LPS) (InvivoGen, Toulouse, France) or were left untreated. Subsequently, cells were harvested and TNF-producing or IL-10-producing monocytes were identified using cytokine secretion assays according to the manufacturer’s protocol (Miltenyi Biotec). To determine IL-23 and IL-1β production, BD GolgiStop was added and intracellular cytokine staining was performed using BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (Becton Dickinson GmbH, Heidelberg, Germany) and anti-IL-23 (eBioscience, San Diego, CA, USA) or anti-IL-1β (R&D Systems, Minneapolis, MN, USA) antibodies. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software.

Reactive oxygen intermediate measurement
PBMCs were stimulated for up to two hours with 100 ng/ml ultrapure LPS or 20 ng/ml phorbol 12-myristate 13-acetate (PMA) or were left untreated. To detect reactive oxygen intermediates (ROIs), dihydrorhodamine 123 was added to the living cells and fluorescence was monitored using a FACSCalibur flow cytometer and CellQuest software.

Statistical analysis
To determine statistical significance, Student’s t-test or the Mann–Whitney rank-sum test was performed. Prior to all comparisons, the Kolmogorov-Smirnov normality test was done.

Results
Frequency of CD56+ monocytes increases throughout life in healthy controls
CD56+ monocytes were identified in PBMCs by the co-expression of CD56 and the monocytic surface marker CD14 (Figure 1a). Eighty-six healthy controls were analyzed, and 8.6% ± 0.6 of the monocytes coexpressed CD56. Most of the CD56+ monocytes are CD14bright (7.9% ± 0.6), and only a minor population is CD14dim (0.7% ± 0.1). Based on the scatter properties in flow cytometric analyses, the CD14bright/CD56+ monocyte subset had the appearance of classical monocytes, whereas the CD14dim/CD56+ monocyte subset resembled large...
lymphocytes and natural killer (NK) cells (data not shown).

All subsequent analyses were performed on CD14^{bright}/CD56+ monocytes in comparison to CD14^{bright}/CD56− monocytes, unless stated otherwise.

It is already known that CD14^{bright} monocytes can be subdivided into two subpopulations by the expression of the surface marker CD16 [1], representing classical monocytes (CD14^{bright}/CD16−) and intermediate monocytes (CD14^{bright}/CD16+). CD16 and CD56 expression was coanalyzed in ten healthy controls. CD56+ monocytes were found to be mostly CD16− and therefore to belong to the classical CD14^{bright}/CD16− monocytes. Only 7.5% ± 1.6 of the CD56+ monocytes coexpressed CD16 and belonged to the intermediate CD14^{bright}/CD16+ monocytes.

To analyze the influence of age on the CD56+ monocyte subpopulation, the control cohort was recruited to reflect a wide age range (median age 53.5 years, range 22 to 72 years). The analysis revealed a close positive correlation between age and CD14^{bright}/CD56+ monocyte frequency (Figure 1b), as well as between age and CD14^{dim}/CD56+ monocyte frequency \((r = 0.332, P = 0.0018)\). No difference in CD56+ monocyte frequencies between women and men were discernible (data not shown).

**CD14^{bright}/CD56+ monocytes show an increased cytokine production and spontaneous reactive oxygen intermediate production**

Little is known about the cytokine production of CD56+ monocytes. Sconocchia et al. performed a cytokine array analysis [2], showing that CD56+ monocytes produce typical monotypic cytokines, but no in vitro studies of cytokine production of CD56+ monocytes in comparison to CD56− monocytes have been performed.

A cytokine secretion analysis in monocytes of young healthy controls revealed that increased frequencies of cells producing the proinflammatory cytokines TNF and IL-23 and the anti-inflammatory cytokine IL-10 in response to LPS were found in CD14^{bright}/CD56+ monocytes compared to CD14^{bright}/CD56− monocytes (Figure 1c). In contrast, intracellular staining for IL-1β showed a similar cytokine production of both CD14^{bright} monocyte subsets in response to LPS (Figure 1c). No difference in the spontaneous production of cytokines was observed (data not shown).

The production of ROIs in response to bacterial products is a characteristic feature of monocytes. No difference in LPS-induced ROI production was observed between CD14^{bright}/CD56+ monocytes and CD14^{bright}/CD56− monocytes (data not shown). The same result was obtained when the production of ROIs was initiated by direct stimulation of protein kinase C with PMA (data not shown). However, the spontaneous production of ROIs was higher in CD14^{bright}/CD56+ monocytes than in CD14^{bright}/CD56− monocytes (Figure 1d).

**Frequency of CD14^{bright}/CD56+ monocytes is increased in young rheumatoid arthritis patients**

To analyze the CD56+ monocyte subpopulation in patients with RA, 75 patients with a median age of 57.0 years (range 23 to 83 years) were recruited. For a detailed clinical description of the patient cohort, see Table 1. Of the RA monocytes, 13.1% ± 0.9 coexpressed CD56, 12.2% ± 0.9 were CD14^{bright} and 0.9% ± 0.1 were CD14^{dim}.

In the global analysis, RA patients had a higher frequency of CD14^{bright}/CD56+ monocytes than healthy controls (12.2% ± 0.9 vs. 7.9% ± 0.5; \(P = 0.0002\)). The CD14^{dim}/CD56+ monocyte subset was also found to be expanded in RA patients (0.9% ± 0.1 vs. 0.7% ± 0.1; \(P = 0.029\)), although the difference was less pronounced. In view of the strong dependence of the CD56+ monocyte frequency on age in the control cohort, the RA patients and controls were separated into three different age subsets (20 to 39 years, 40 to 59 years and 60 years and older). As shown in Figure 2a, the increase of CD14^{bright}/CD56+ monocyte frequencies in the RA patients was limited to the age subset from 20–39 years, while frequencies in older RA patients did not differ significantly from age-matched controls. The CD14^{dim}/CD56+ monocyte subset was also found to be expanded in young RA patients (0.6% ± 0.1 vs. 0.8% ± 0.1, \(P = 0.046\)) but not in older patients (data not shown). In the global analysis of the total RA cohort, no correlation of age with the CD14^{bright}/CD56+ monocyte subset was found (Figure 2b).

Analysis of the disease duration prior to study enrollment revealed no correlation with the CD14^{bright}/CD56+ monocyte frequency (data not shown). The CD14^{bright}/CD56+ monocyte subset is already expanded early in the disease because RA patients with a disease duration of one year have 12.0% ± 1.8 CD14^{bright}/CD56+ monocytes compared to 12.3% ± 1.1 CD14^{bright}/CD56+ monocytes in patients with a longer disease duration (\(P = \text{n.s.}\)). The therapeutic regimen at the time of the analysis, the presence of rheumatic factor or anti-CCP antibodies, the absolute monocyte count, C-reactive protein levels and gender had no influence on CD14^{bright}/CD56+ monocyte frequency.

**Therapeutic tumor necrosis factor blockade with etanercept decreases frequency of CD14^{bright}/CD56+ monocytes**

To analyze the influence of treatment and of the therapeutic response on the frequency of the expanded CD14^{bright}/CD56+ monocyte subset, the cell population
was quantified in a longitudinally followed cohort of 16 RA patients before and during 24 weeks of treatment with etanercept. Before the start of treatment, RA patients had a mean CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte frequency of 12.4% ± 1.7, and no correlation of disease activity with the frequency of CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes was detectable (data not shown).

In the prospective analysis, the frequency of CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes in this patient cohort was found to decrease after eight weeks of treatment and remained decreased for up to twenty-four weeks, which was the endpoint of the study (Figure 3a). Interestingly, RA patients with a good response to the treatment also showed a more pronounced decrease in the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte frequency after 12 weeks (Figure 3b) and 24 weeks (data not shown) of treatment.

Discussion

To date, the CD56\textsuperscript{+} monocyte subpopulation has not been very well-characterized. Although CD56\textsuperscript{+} monocytes were found in 2001 in patients with Down syndrome with or without an additional hematological disorder [4], Sconocchia et al. first described CD56\textsuperscript{+} monocytes in the peripheral blood of healthy controls in detail in 2005 [2]. However, the same study group had previously described the differentiation of CD56\textsuperscript{+} monocytes from CD34\textsuperscript{+} stem cells in 2004 [11]. Herein we show that the frequency of CD56\textsuperscript{+} monocytes dramatically increases with age in healthy controls; that CD56\textsuperscript{+} monocytes produce more TNF, IL-10 and IL-23; that CD56\textsuperscript{+} monocytes are expanded in young RA patients; and that the CD56\textsuperscript{+} monocyte subset responds to anti-TNF treatment in RA patients.

We were able to identify two subsets of CD14\textsuperscript{+} monocytes coexpressing CD56 in the peripheral blood of healthy controls and RA patients. The CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset represents the main CD56\textsuperscript{+} monocyte population, and these cells had the same flow cytometry scatter appearance as classical monocytes. In contrast, the minor CD14\textsuperscript{dim}/CD56\textsuperscript{+} monocyte subset mostly had the appearance of lymphocytes and NK cells, which probably corresponds to the CD56\textsuperscript{+} cell population described by Gruenbacher et al. [12]. This group described CD56\textsuperscript{+} cells in the peripheral blood, which are CD14\textsuperscript{dim}, HLA-DR\textsuperscript{+} and CD86\textsuperscript{+}; have the appearance of intermediate-sized lymphocytes; and differentiate \textit{in vitro} into dendritic cells. Sconocchia et al. also described a CD56\textsuperscript{+}/CD33\textsuperscript{+} myeloid cell population which is able to differentiate \textit{in vitro} into

![Figure 2 CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset is expanded in young rheumatoid arthritis patients. (a) Peripheral blood frequencies of CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes in healthy controls (HD) and rheumatoid arthritis patients (RA). (b) Correlation between age and the peripheral blood frequencies of CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes in RA patients.](image-url)
cells with a dendritic cell morphology [13]. Although the two CD14\textsuperscript{bright}/CD56\textsuperscript{+} and CD14\textsuperscript{dim}/CD56\textsuperscript{+} populations have different size and granularity proportions, it remains to be established whether these populations are truly two different monocyte subsets or if they belong to one CD56\textsuperscript{+} monocyte subset. Some of our observations point to the latter possibility because both CD14\textsuperscript{bright}/CD56\textsuperscript{+} and CD14\textsuperscript{dim}/CD56\textsuperscript{+} populations increase comparably with age in healthy controls and are equally expanded in RA patients.

We observed only a minimal overlap of the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset with the well-characterized CD14\textsuperscript{bright}/CD16\textsuperscript{+} intermediate monocyte subset (7.5% of the CD56\textsuperscript{+} monocytes coexpressed CD16). Contamination with NK cells can be excluded because the cells expressed high levels of the monocytic lineage marker CD14. CD14\textsuperscript{bright}/CD16\textsuperscript{+} intermediate monocytes are the main producer of IL-1β, TNF and IL-23 in response to LPS [14]; are expanded in RA patients [6]; and promote the expansion of Th17 cells [6]. Most of the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes belong to the classical CD14\textsuperscript{bright}/CD16\textsuperscript{−} monocyte subset, which is the main producer of ROIs, CCL2 and IL-8 in response to LPS [14]. In our study, we demonstrated that CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes produce more of the typical monocyte cytokines TNF, IL-10 and IL-23 in response to LPS than CD14\textsuperscript{bright}/CD56\textsuperscript{−} monocytes do. This cytokine response to LPS classifies the CD14\textsuperscript{bright}/CD56\textsuperscript{−} monocytes into the inflammatory subset described by Cros et al., contrary to the patrolling monocytes which respond to viruses and nucleic acids, but only weakly to LPS [14].

One major finding of the present study is the strong expansion of the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset with increasing age in healthy controls. The aging process is associated with age-related changes in the immune system, a phenomenon called immunosenescence [15,16]. In RA, characteristic signs of immunosenescence are present in the adaptive immune system, in particular in the T-cell compartment [17]. However, immunosenescence can also be observed in the innate immune system [18]. Monocytic cells of aged mice produce lower amounts of cytokines in response to Toll-like receptor activation [19,20], but older mice have increased cytokine levels in in vivo models of sepsis [21,22]. The influence of age on LPS-induced human monocyte cytokine production has been a subject of controversy, with reports ranging from increased to decreased cytokine production [23-26]. In our study, the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocytes produced more cytokines in response LPS and spontaneously released more ROIs than classical monocytes, but more comprehensive analyses in older people are needed.

Conflicting results have been reported regarding the influence of age on absolute monocyte numbers in the peripheral blood [27,28] and the CD16 monocyte subset composition [23,28,29]. In a previous study of the CD14\textsuperscript{bright}/CD16\textsuperscript{+} monocyte subset in patients with RA, however, we did not observe an age-dependent increase in the CD16\textsuperscript{+} monocyte subsets in healthy controls and RA patients [6]. In the present study, we observed an increase in the CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset from 4.7% in healthy controls younger than 30 years of age to 10.2% in healthy controls older than 60 years of age. The CD14\textsuperscript{bright}/CD56\textsuperscript{+} monocyte subset in RA is expanded in younger patients, probably reflecting a preaged innate immune system in RA patients.

In RA, the monocyte subset is dysregulated. In comparison to healthy controls, the absolute number of all monocyte subsets is increased [7,30], and, in relation to the other CD16-defined monocyte subsets, the CD14\textsuperscript{bright}/CD16\textsuperscript{+} monocyte subset is also expanded [6]. Herein we
describe another monocyte subpopulation, CD14$^{bright}$/CD56$^+$/CD56$^-$ monocytes, which is expanded in RA patients. Considering that the CD14$^{bright}$/CD56$^+$ monocyte subset only minimally overlaps the CD14$^{bright}$/CD16$^+$ monocyte subset, RA patients have a major shift toward pathological monocyte subpopulations at the expense of classical monocytes. The expansion of the CD14$^{bright}$/CD56$^+$ monocyte subset in RA patients was not associated with the inflammatory state of the patients, but we did observe a reduction of the subpopulation during anti-TNF treatment. This decrease in the CD14$^{bright}$/CD56$^+$ monocyte frequency was also associated with a better response to the treatment.

**Conclusion**

The CD14$^{bright}$/CD56$^+$ monocyte subset is present in healthy controls and expands with increasing age. The frequency of CD14$^{bright}$/CD56$^+$ monocytes is increased in RA patients, declines with effective anti-TNF treatment and is associated with a better response to treatment. CD14$^{bright}$/CD56$^+$ monocytes produce increased cytokine levels in response to LPS and have higher spontaneous ROI production. The CD14$^{bright}$/CD56$^+$ monocyte subset might therefore represent monocytes from a preaged immune system with dysregulated cytokine and ROI production, but further studies are required to confirm this hypothesis.

**Abbreviations**

LPS: Lipopolysaccharide; RA: Rheumatoid arthritis; ROI: Reactive oxygen intermediate; TNF: Tumor necrosis factor.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

MK performed most of the experiments and was involved in data analysis. CB and UW were involved in the study design and data analysis. MR conceived of the project, was involved in the study design and data analysis, and drafted the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Cornelia Arnold for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft DFG (RO 4037/1-1).

**References**


doi:10.1186/ar4321
Cite this article as: Krasselt et al.: CD56+ monocytes have a dysregulated cytokine response to lipopolysaccharide and accumulate in rheumatoid arthritis and immunosenescence. Arthritis Research & Therapy 2013 15:R139.
Dissertation

zur Erlangung des akademischen Grades Dr. med.

CD56\(^+\) Monocytes Have a Dysregulated Cytokine Response to LPS and Accumulate in Rheumatoid Arthritis and Immunosenescence

eingereicht von: Marco Lothar Krasselt
angefertigt am / in: 3. Februar 2014, Universitätsklinikum Leipzig
betreut von: Prof. Dr. med. Ulf Wagner, Dr. rer. nat. Manuela Rossol
Monat und Jahr: Februar 2014

IV. Summary

Peripheral blood monocytes are no longer regarded as a homogeneous cell population, but can be differentiated into various subpopulations. In the present study, CD14\(^{\text{bright}}\)/CD56\(^+\) monocytes, an only recently identified and poorly examined subpopulation, was further investigated. This population had been described before, but the current investigations yielded the following new findings:

1. a higher frequency of those cells among patients suffering from Rheumatoid Arthritis (RA), especially in young patients below the age of 40,
2. a strong positive correlation of the frequency of this subpopulation with the age of the healthy controls,
3. an increased cytokine and reactive oxygen intermediate (ROI) production of CD14\(^{\text{bright}}\)/CD56\(^+\) monocytes compared to classical monocytes and
4. a reduction of those cells during effective TNF\(\alpha\)-blocking treatment.

This findings are of great interest and implicate these cells in the complicated pathogenesis of RA, but also in immunosenescence.
Flow Cytometry Results

Healthy controls
Overall 86 healthy donors (22-72 years old, 53.5 years in median) were included and blood samples have been processed for flow cytometry, in order to stain PBMCs for the fluorescent markers of interest (anti-CD56-APC, anti-CD14-FITC and anti-CD16-PE). CD56+ monocytes were identified in PBMCs by the co-expression of CD56 and the monocytic surface marker CD14. Based on the scatter properties in flow cytometric analyses, the CD14bright/CD56+ monocyte subset had the appearance of classical monocytes; the CD14dim/CD56+ ones rather resemble lymphocytes and NKCs. Most of the cells of our interest belong to the typical CD14bright/CD16− monocyte subset.

Results showed a strong positive correlation of CD14bright/CD56+ monocytes and the donor’s age (r=0.563; p < 0.0001) without any differences between men and women.

RA patients
Overall 75 RA patients (23-83 years old, 57 years in median) were included and blood samples have been processed just like for the healthy donors. Results showed a higher frequency of CD14bright/CD56+ monocytes in patients than in healthy controls (12.2 % ± 0.9 vs. 7.9 % ± 0.5; p=0.0002) with no age correlation at all.
When dividing all donors, patients and controls, into three age-matched groups (20-39 yrs., 40-59 yrs., 60 yrs. and above), the increased frequency favouring the patients only was to be found within the youngest group (20-39 yrs.) while not seeing any significant differences at the age of 40 and above.

Cytokine Production and Reactive Oxygen Intermediate Measurement
Investigations further revealed CD14bright/CD56+ monocytes showing an increased cytokine production on stimulation with ultrapure lipopolysaccharide (LPS) as well as an increased spontaneous reactive oxygen intermediate (ROI) production. All analyses were performed on monocytes of healthy controls using cytokine secretion assays, intracellular cytokine staining or dihydrorhodamine 123-based ROI-measurement respectively.

Cytokine production
The significantly increased amount of cells producing cytokines on LPS-stimulation was found for the pro-inflammatory cytokines TNFα and IL-23; the frequency was increased for IL-10 as well. There was no significant difference in IL-1β production.

ROI production
Contrary, when examining ROI production, no difference after LPS or PMA stimulation was found while the spontaneous production was significantly increased in CD14bright/CD56+ monocytes.
Longitudinal Etanercept Study

To analyze the influence of treatment and the therapeutic response on the frequency of the expanded CD14$^{\text{bright}}$/CD56$^+$ monocyte subset, the cell population was measured in a longitudinally, prospective treatment study using Etanercept. The cohort consisted of 16 RA patients.

The median amount of the CD14$^{\text{bright}}$/CD56$^+$ monocyte subset decreased significantly during the therapy with Etanercept after 8 weeks and remained low for the whole study duration of 24 weeks. Additionally and clinically very important, the decrease in CD14$^{\text{bright}}$/CD56$^+$ cells went along with a good treatment response measured by the clinical widely used Disease Activity Score 28 (DAS28).

Conclusion

The results of the study presented in the published manuscript are promising and very interesting when trying to understand the pathogenesis of RA. The described CD14$^{\text{bright}}$/CD56$^+$ monocyte subset is not only positively correlated with age in healthy controls but also seems to be increased in young patients suffering from RA. These findings alone are leading to the assumption, that there might be a connection to immunosenescence, possibly with relevance for the development of RA.

The aging of the human immune system was described and investigated before, more widely for the adaptive, but also for the innate immunity. Results remain conflicting so far, but there is consensus about an inflammatory condition, called “inflame-aging” in older people as being illustrated by elevated cytokine levels.

Especially the age-dependent response of monocytes to LPS-activation is discussed controversially, with results ranging from decreased to increased cytokine production. The results at hand show an increased production of pro-inflammatory cytokines within the investigated subpopulation, supporting the assumed immunosenescent state in the elderly. This assumption is emphasized by the fact, that spontaneous ROI production occurs more frequently. It seems that RA patients are having a disturbed monocyte panel with an increased amount of pro-inflammatory monocytes at the expense of classical monocytes.

Moreover, the frequency of the examined subpopulation in peripheral blood declines with effective TNFα-blocking treatment and this decline is associated with a better therapy response.

When summing up all those findings, the identified monocyte subset might represent cells of a prematurely aged immune system with a dysregulated cytokine production pattern and an increased spontaneous ROI production, which are probably involved in the pathogenesis of Rheumatoid Arthritis. Additional investigations are needed to confirm those hypotheses.
V. Enclosures
V.1 Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ACPA</td>
<td>Anti-citrullinated Proteins/Peptides Antibodies</td>
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<tr>
<td>APC (I) [FACS]</td>
<td>Allophycocyanin</td>
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<td>APC (II)</td>
<td>Antigene Presenting Cell</td>
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<td>BCR</td>
<td>B cell Receptor</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CR</td>
<td>Complement Receptor</td>
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<td>DAS</td>
<td>Disease Activity Score</td>
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<td>FACS</td>
<td>Fluorescence-activated Cell Sorting</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>FoxP3</td>
<td>Forkhead-Box-Protein P3</td>
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<td>HLA</td>
<td>Human Leucocyte Antigen</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<tr>
<td>NCAM</td>
<td>Neural Cell Adhesion Molecule</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor 'kappa-light-chain-enhancer' of Activated B-cells</td>
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<tr>
<td>NKC</td>
<td>Natural Killer Cell</td>
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<tr>
<td>PADI</td>
<td>Peptidylarginine Deiminase</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononucleated Cell</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PMA</td>
<td>Phorbol-12-myristat-13-acetat</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear Neutrophil</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein Tyrosine Phosphatase, Non-Receptor type 22</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>RF</td>
<td>Rheumatoid Factors</td>
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<td>ROI</td>
<td>Reactive Oxygen Intermediate</td>
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<td>RR</td>
<td>Relative Risk</td>
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<td>SE</td>
<td>Shared Epitope</td>
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<td>Single Nucleotide Polymorphism</td>
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<td>T cell Receptor</td>
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<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
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<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor α</td>
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V.2 Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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Ort, Datum ........................................ M. L. Krasselt
V.3 Curriculum Vitae
V.4 Publications

Original Research Article
Impact Factor: 4.302

Review
Impact Factor: 0.653
V.5 Closing Words

This thesis is dedicated to my marvelous mother Andrea (1962-1998) who left way before her time. You made me become a physician and a better person.

Completing it was only possible due to the awesome support of my loving family, especially my beloved Julia, my wonderful sister Katrin and my great father Gunther. It is you guys overwhelming me with gratitude for your help. I honestly thank you for always being there and never stopping to believe in me.

I also sincerely thank Dr. Manuela Rossol and Prof. Dr. Ulf Wagner for giving me the oppurtunity to do this work using their lab and beginning to research on those huge and very interesting field of science. It has been a great pleasure to work with you both; you have always had an opened ear for me and, more important, I could count on your personal support as well as your scientific expertise anytime.

Last but not least I want to thank the Medical Faculty of the University of Leipzig for granting the urgently needed scholarship for this work when I still was attending medical school.

People are always blaming their circumstances for what they are. I don’t believe in circumstances. The people who get on in this world are the people who get up and look for the circumstances they want, and, if they can’t find them, make them.

George Bernhard Shaw