Exploring the world of non-coding genes in stem cells and autoimmunity.

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Chapter 2

Inflammatory genes TNFα and IL6 display no signs of increased H3K4me3 in circulating monocytes from untreated rheumatoid arthritis patients

Genes Immun. 2017 Sep;18(3):191-196. doi: 10.1038/gene.2017.20.

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Abstract

Innate immune cells, such as monocytes, can adopt a long-lasting pro-inflammatory phenotype, a phenomenon called 'trained immunity'. In trained immunity, increased cytokine levels of genes, like interleukin (IL)-6 and tumor necrosis factor (TNF)-α, are observed, which are associated with increased histone 3 lysine 4 trimethylation (H3K4me3) in the promoter region. As systemic IL6 and TNFα levels are increased in rheumatoid arthritis (RA) patients and monocytes are known to be the primary producers of TNFα and IL6, we hypothesized that 'trained immunity' signals may be observed at these genes in monocytes from RA patients. CD14+ monocytes were isolated from untreated RA patients and paired age-matched healthy controls. H3K4me3, mRNA, protein and serum levels of IL6 and TNFα were evaluated by chromatin immunoprecipitation, reverse-transcription quantitative PCR and enzyme-linked immunosorbent assays. Despite elevated serum levels of TNFα and IL6 in the tested RA patients (P<0.05), *ex vivo* isolated monocytes displayed similar H3K4me3 levels to healthy controls in the promoter region of TNFα and IL6. Concordantly, mRNA and protein levels of IL6 and TNFα were similar before and after lipopolysaccharide stimulation between patients and controls. Together, with the current number of individuals tested we have not detected enhanced trained immunity signals in circulating monocytes from untreated RA patients, despite increased IL6 and TNFα serum levels.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease mainly characterized by inflammation of the joints. Although some effective treatment of RA exists, many factors that lead to the development of the disease remain unknown. Both environmental and genetic factors have been shown to have a role in the onset of the disease.[1] For many diseases, including those of autoimmune nature, environmental triggers are thought to influence gene regulation via changes in the epigenetic landscape.[2,3] Epigenetic changes are defined by non-genetic changes in gene activity, which are influenced by various factors including the following: non-coding RNAs, DNA methylation and histone modifications.[4,5] In innate immune cells as well, environmental triggers were shown to induce long-lasting epigenetic changes described in a concept known as 'trained immunity'.[6] In the study from Quintin *et al.*,[6] monocytes were pre-exposed to β-glucan (a cell-

wall component of *Candida albicans*) for 24 h, which resulted in changes of the epigenetic landscape. One of the identified hallmarks of trained immunity was an induction of histone 3 lysine 4 trimethylation (H3K4me3) in the promotor region of immune genes like tumor necrosis factor (TNF)-α and interleukin (IL)-6, an indicative mark for active promoters.[6,7] As a result of increased H3K4me3 levels, a second immune stimulation of these pre-exposed monocytes resulted in an increased immune response shown initially by elevated IL6 and TNFα levels.[6] These results indicated that innate immune cells like monocytes can acquire long-lasting alterations in the epigenetic landscape resulting in the enhanced production of cytokines.

In RA, TNFα and IL6 are elevated in the serum and synovium of patients compared with healthy individuals.[8] Moreover, both anti-TNFα treatment and anti-IL6 treatment are currently successful therapies against RA, suggesting the importance of both cytokines in disease pathogenesis.[9] As monocytes can produce high levels of TNFα and IL6, we hypothesized that changes in H3K4me3 of monocytes might exist in early untreated RA patients, thereby contributing to enhanced cytokine levels and thus to the disease pathogenesis of RA.[10] To our knowledge, this is the first report measuring H3K4me3 levels in the promoter region of two RA-relevant cytokines in primary immune cells.

Results

Detection of differences in H3K4me3 levels in the promoter of IL6 and TNFα in human monocytes

In order to test our hypothesis and to show that in our hands monocytes can obtain a trained phenotype, we repeated the previously published trained immunity model as described by Quentin *et al.*[6] Monocytes from healthy donors were pre-incubated with β-glucan for 24 h followed by a 6-day resting/refreshing period. After 7 days, H3K4me3, protein and RNA levels of TNFα and IL6 from these monocytes were compared with monocytes, which were not exposed to β-glucan from the same donor. We observe similar effects with a ~2-fold increase in H3K4me3 levels at the TNFα and IL6 promoter regions with a concurrent increase in RNA and cytokine levels (Supplementary Figure S1). We next calculated how many RA patients and matched healthy controls we would require to detect

similar effect sizes. A total sample size of four is required, two RA patients and two healthy controls, providing 80% to detect a significant difference at $P<0.05$.

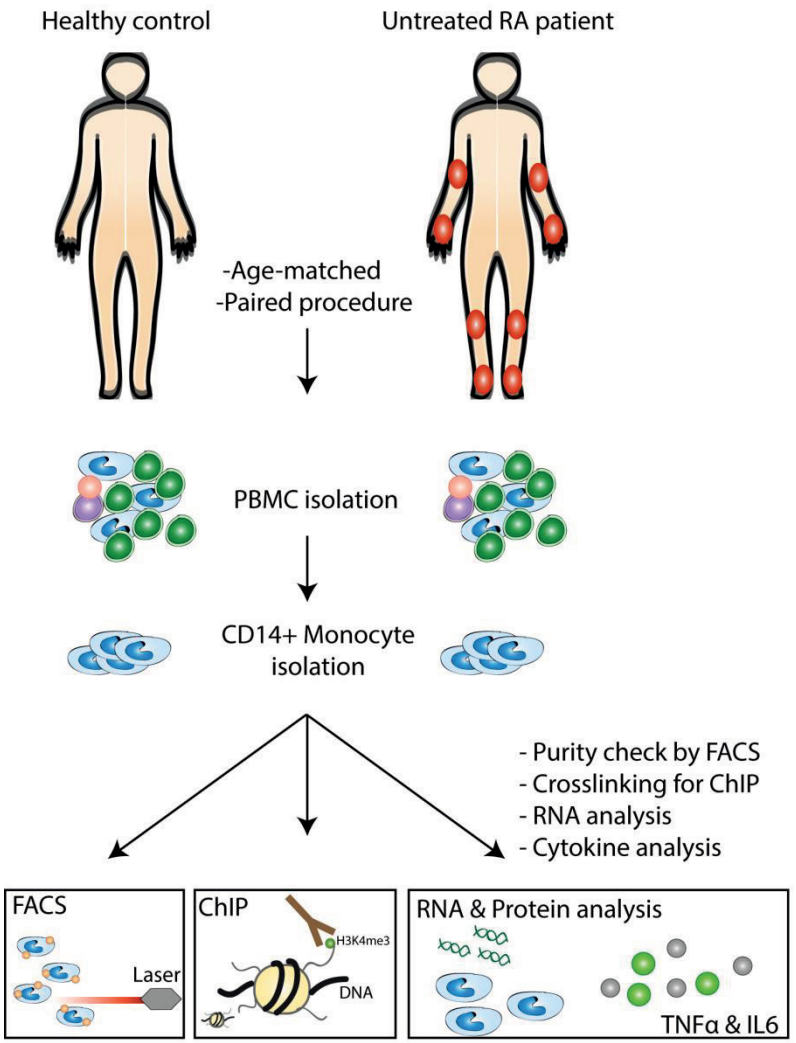

Figure 1. Flowchart of technical procedure isolating monocytes from healthy controls and untreated RA patients. Blood was obtained from a newly diagnosed untreated RA patient and a healthy control on the same day and used for PBMCs isolation. After a paired PBMC isolation, CD14+ monocytes were isolated by magnetic beads and used for (i) purity check by fluorescence-activated cell sorting, (ii) DNA-Histone crosslinking and stored at −80 °C for ChIP experiments or (iii) RNA and protein analysis by culturing the isolated monocytes in presence of RPMI (unstimulated) or LPS.

Similar H3K4me3 levels on the IL6 and TNFα promoter in monocytes from RA patients and healthy controls

To minimize sources of variation between patients and controls, we simultaneously collected blood and serum from RA patients and age-matched healthy individuals (Figure 1 and Supplementary Figure S2). RA patients display significantly higher IL6 and TNFα levels in serum as compared with controls, indicating that *in vivo* differences in cytokine levels are present (Supplementary Figure S3). In order to measure H3K4me3 levels, CD14+ monocytes were directly isolated and H3K4me3 levels of TNFα and IL6 were simultaneously measured. However, H3K4me3 levels on the promoter region of TNFα and IL6 were similar in untreated RA patients and healthy controls (Figures 2a and b, left panel, *P*=0.61 for both genes). The observed content of H3K4me3 from our experiments coincide with previously measured H3K4me3 chromatin immunoprecipitation (ChIP)-sequencing levels in healthy individuals derived from publicly available data from ENCODE/broad institute (Figures 2a and b, right panel). H3K4me3 levels of housekeeping gene RPL5 were similar between untreated RA patients and healthy controls, and correcting for RPL5 as an additional internal control did not alter the H3K4me3 levels between healthy controls and untreated RA patients (Supplementary Figure S4A and B). Finally, Insulin, which is not expressed by monocytes, was used as a negative control and did not display H3K4me3 levels in monocytes as expected (Supplementary Figure S4C).

Similar IL6 and TNFα mRNA and protein levels, and responsiveness of monocytes from RA patients and healthy controls

To confirm our histone trimethylation results and further confirm that both monocytes from RA patients and healthy controls respond similarly, we evaluated RNA levels of TNFα and IL6 before and after lipopolysaccharide (LPS) stimulation. In unstimulated monocytes we observed low levels of TNFα and IL6 that were similar in controls and RA patients (Supplementary Figure S5). Upon stimulation, a strong induction of expression was observed in both healthy controls and RA patients for TNFα and IL6 (Figures 3a and b). Again, similar RNA levels between both groups were observed for IL6 (RA: 42637±19208 vs controls: 47996±38235; *P*=0.70) and TNFα (RA: 78.5±20.9, controls: 83.5±42.3; *P*=0.9). In addition, we investigated cytokine levels in the supernatant of unstimulated and LPS-stimulated monocytes. Similar protein levels were observed in the

supernatant of monocyte cultures obtained from the two groups (Figures 3c and d), although TNFα and IL6 levels seemed slightly elevated in RA patients after LPS-stimulation (TNFα concentration: RA: 4.3 ng ml^{-1} ±2.9 vs controls: 2.8 ng ml^{-1} ±2.6; *P*=0.40); IL6 concentrations: RA: 67.2 µg ml^{-1} ±14.6 vs controls: 50.8 µg ml^{-1} ±17.3; *P*=0.19). In addition, dividing patients on basis of autoantibody status (anti-citrullinated protein antibody positivity) revealed neither differences in IL6 and TNFα production (Supplementary Figure S6). Together, these findings suggest that there are no substantial differences regarding H3K4me3, mRNA levels or cytokine levels of IL6 and TNFα between circulating monocytes from RA patients and healthy controls in this study.

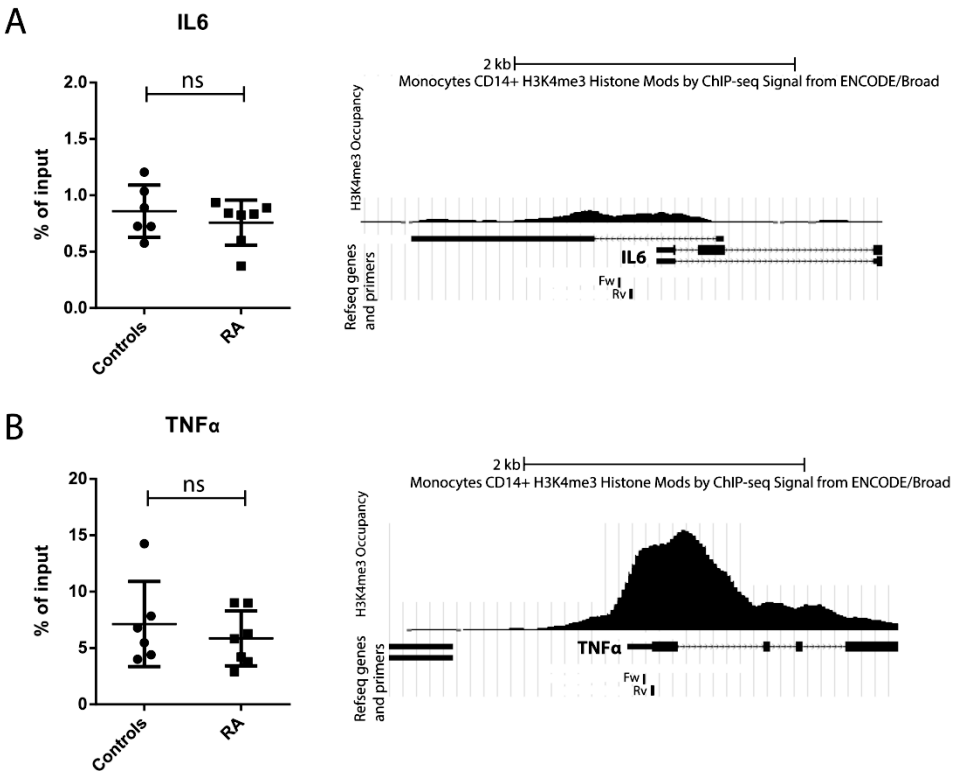

Figure 2. H3K4me3 levels in monocytes from healthy controls and untreated RA patients. (a) H3K4me3 levels of the IL6 promoter in healthy controls (controls) and untreated RA patients (RA) (top). The y axis represents the percentage of precipitated H3K4me3 normalized for DNA input. H3K4me3 occupancy of the IL6 promoter in healthy individuals measured by ChIP-sequencing (ChIP-seq) from ENCODE/Broad institute (bottom). (b) H3K4me3 levels of the TNFα promoter in healthy controls (controls) and untreated RA patients (RA) (top). H3K4me3 occupancy of the TNFα promoter in healthy individuals measured by ChIP-seq from ENCODE/Broad institute (bottom). Mann–Whitney *U*-test was used to test for significant differences.

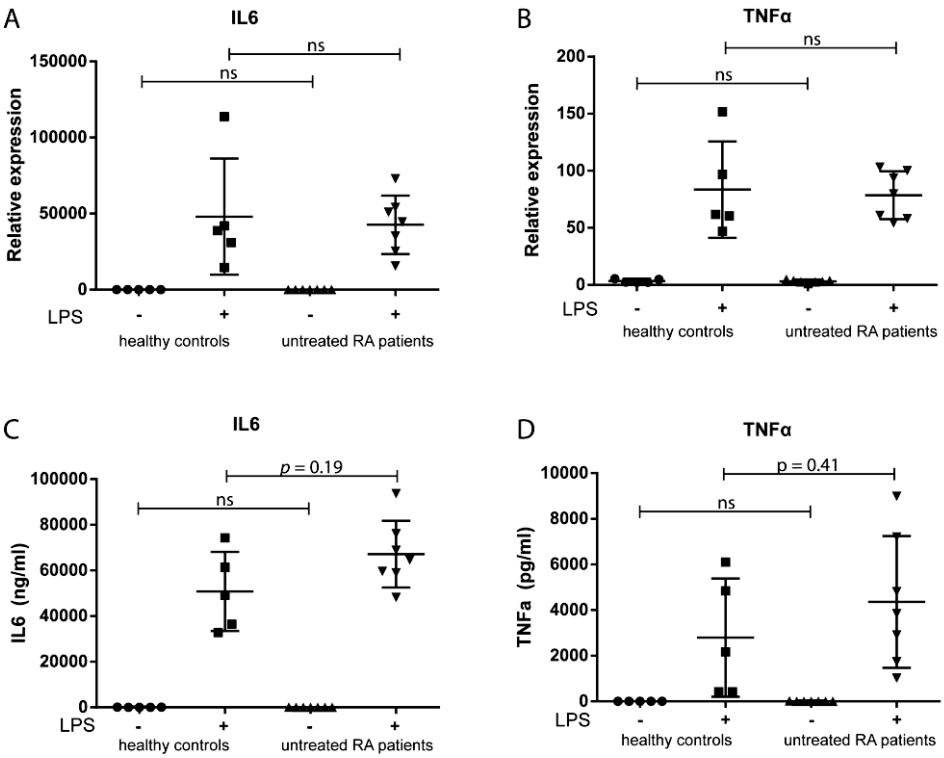

Figure 3. RNA and protein levels of IL6 and TNFα in monocytes from healthy controls and RA patients. RNA levels of IL6 (a) and TNFα (b) were determined for unstimulated (−) and LPS-stimulated (+) monocytes isolated from healthy controls and untreated RA patients. RNA levels were normalized for *GAPDH* and *B2M* as housekeeping genes. The sample with the lowest expression was set to 1. Protein concentrations of IL6 (c) and TNFα (d) were measured in the supernatant of unstimulated (−) and LPS-stimulated (+) monocytes isolated from healthy controls and untreated RA patients. The concentration of LPS added was 10 ng ml^{-1}. Mann–Whitney *U*-test was used to test for significant differences.

Discussion

In this study we aimed to measure H3K4me3 levels in the promotor regions of TNFα and IL6, in *ex vivo* primary monocytes from RA patients and healthy individuals. TNFα and IL6 are important cytokines for the pathogenesis of RA and can both be found in large abundances in the joints of RA patients.[11,12] IL6 can activate several cell types and is involved in the maturation of B cells and thus the production of autoantibodies.[13] TNFα is a potent inducer of other inflammatory

genes and inhibition of either TNFα or IL6 have both been shown to be effective for treatment of RA.[14,15] Monocytes are the primary producers of IL6 and TNFα and 'trained' monocytes have been shown to have enhanced production of both cytokines. In order to test whether monocytes from RA patients display different epigenetic patterns at these two genes, a rigorous protocol of collection of blood and isolation of monocytes from both RA patients and paired age-matched healthy controls was implemented. To our knowledge, this is the first time that a study addressing epigenetic changes in *ex vivo* primary monocytes from early untreated RA patients has been performed.

Epigenetic modifications have a crucial role in regulating gene expression.[16] The role of DNA methylation and histone modifications have both been studied in relation to RA.[17] In synovial fibroblast, structural changes in DNA methylation correlate with fibrotic development in the joint of RA patients.[18] In several immune cells as well, changes were observed in the DNA methylation of relevant immune-related genes, for example, hypomethylation of IL6 in B cells of RA patients[19] or hypomethylation of CD40LG in CD4+ T cells.[20] Furthermore, expression levels of various histone-modifying enzymes have been investigated in RA. EZH2 (Enhancer of Zeste Homolog 2), a histone methyl transferase was found elevated in synovial fibroblast from RA patients.[21] However, studies evaluating changes in histone methylation in patients with autoimmune diseases are scarce. In this study, we evaluated for the first time H3K4me3 levels on the promoter of two important cytokine genes in monocytes directly isolated from RA patients.

Although many types of histone modifications are known, H3K4me3 was shown to correlate with higher TNFα and IL6 levels in β-glucan-trained monocytes and was therefore used as a marker for trained immunity. Here we report that although we can reliably measure twofold differences in H3K4me3 levels between β-glucan-trained and untrained monocytes, our study was sufficiently powered to detect effect size differences as low as 1.8; however, such epigenetic differences on the H3K4me3 mark between RA patients and healthy controls were not observed. This finding was confirmed by RNA and protein levels of IL6 and TNFα, which had similar levels in RA patients and healthy controls after LPS stimulation. These results indicate that RA monocytes are likely not in a continuous pro-inflammatory ON-state at the IL6 and TNFα genes on basis of the H3K4me3 levels and LPS responsiveness. Although we demonstrate that *in vitro*

trained monocytes have similar time-response dynamics in their cytokine production as untrained monocytes (Supplementary Figure S7), we cannot exclude that RA-specific differences in cytokine dynamics exist. It is probable that RA monocytes produce higher TNFα and IL6 levels *in vivo* via other mechanisms as the possibility exists that RA monocytes will stay activated longer compared to healthy controls and such effects will be missed in our study due to the chosen time points and LPS concentration. Moreover, in our study, monocytes were obtained from peripheral blood and we cannot exclude that locally activated monocytes in the joints of RA patients show changes in their epigenetic landscape. Besides monocytes other RA-relevant cells types are capable of producing TNFα and/or IL6. These cell types include T cells, B cells, mast cells, synovial fibroblasts and osteoblasts, and may also contribute to the induced TNFα and IL6 levels in the serum of patients.[22] It would be interesting to investigate whether these cell types can also obtain a trained epigenetic landscape and whether this contributes to development of RA.

Besides changes in H3K4me3 upon immune stimulation, other histone marks such as H3K4me1 and H3K27Ac have more recently been described to shape the trained immunity epigenetic landscape.[23] In line with these findings, latent enhancers also acquire changes in H3K4me1, H3K4me3, H3K27Ac and PU.1 binding upon immune triggering.[24,25] These studies illustrate the complexity of the epigenetic landscape that is formed upon different immune stimuli and suggests that further in-depth studies may be required. Nonetheless, epigenetic changes are currently being used for diagnostics and therapy in other diseases (reviewed in [ref. 26]). In RA as well, an inhibitor of histone-modifying enzyme was suggested as an inflammatory suppressor.[27] Gillespie *et al.*[27] showed that RA peripheral blood mononuclear cells (PBMCs) possess increased histone deacetylase activity and inhibition of this class of enzymes could reduce the production of both TNFα and IL6 in a compound and cell-type-dependent manner.[27] However, PBMCs are a collection of diverse cell types and results obtained represent an average across all cells. These data highlight the complexity and variety of factors that can influence the epigenetic landscape. Investigating the epigenetic landscape in disease pathology is thus influenced by these factors and the outcome of results may rely on (i) the histone modification that is being studied, (ii) the genes investigated and (iii) the choice of cell type. Currently, our results indicate that large differences between circulating

monocytes isolated from RA patients and healthy individuals are absent in context of TNFα and IL6 levels. However, we cannot exclude that more subtle differences may be detected in larger sample sizes. In conclusion, with the current number of individuals tested we have not detected evidence that H3K4me3 levels at two immune relevant genes IL6 and TNFα are enhanced in RA. Further studies addressing genome-wide patterns of epigenetic changes in larger numbers of individuals may yield additional insight into whether epigenetic changes in monocytes may be relevant for the pathogenesis of RA.

Materials and methods

Patients

Patients fulfilling the 1987 criteria for RA[28] recruited from the rheumatologic outpatient clinic of the Leiden University Medical Center were included in this study. Patients were studied at the time of diagnosis and before disease-modifying antirheumatic drugs (including no corticosteroids) were started. Age-matched healthy controls were also included, and were preferably healthy partners or family member of the patients. This study was approved by the ethical committee of the Leiden University Medical Center and all patients and healthy donors provided written informed consent. Serum from patients and healthy controls was obtained by incubation of blood at room temperature for 30 min followed by centrifugation at 2000 *g* for 10 min. Supernatant (serum) was transferred and stored at −80 °C until Illuminex measurements. PBMCs isolations were performed from an untreated RA patient and a matched healthy control and were performed on the same day in a paired manner, with the exception one RA patient, which did not have a paired control. PBMC isolations were performed from eight untreated RA patients (seven females, one male mean±s.d. age 59.3±11.2 years) and seven healthy controls (five females, two males; age 51.6±10.9 years). Four patients were positive for anti-citrullinated protein antibodies and five patients were positive for rheumatoid factor. More detailed characteristics can be found in Supplementary Table S1.

Cell culture

PBMCs from healthy controls and RA patients were isolated on a Ficoll gradient (Pharmacy LUMC, Leiden, The Netherlands). CD14+ monocytes were isolated using magnetic beads and cultured as previously described.[29] Cells were either

crosslinked for ChIP experiments and/or cultured for RNA and protein measurements. For the ChIP experiment, seven RA patients and six healthy controls were used. For seven RA patients and five healthy controls, enough cells were obtained from CD14+ isolation and additional RNA and protein analysis was performed. RNA was isolated 4 h after stimulation with either LPS or RPMI (unstimulated). Supernatant was collected 20 h after stimulation with LPS or from unstimulated monocytes and used for enzyme-linked immunosorbent assay to measure the level of secreted cytokines. β-Glucan stimulation was performed as described by Quintin *et al.*[6] In short, CD14+ isolated monocytes were once pre-incubated for 24 h with either β-glucan (Invivogen, San Diego, CA, USA) from *Alcaligenes faecalis* (10 μg ml^{-1}) or RPMI medium (unstimulated). After 24 h, β-glucan was removed by refreshing with new medium without β-glucan and was continued to be refreshed every 2 days over a 6-day period. After 7 days (1 day β-glucan, 6 day washing/refreshing), monocytes were either used for chromatin immune precipitation or stimulated with LPS (10 ng ml^{-1}) and RPMI (unstimulated) for RNA and protein analysis. Viability of monocytes after 7 days of culturing was monitored by a LUNA-II automated cell counter (Logos Biosystems, Villeneuve d'Ascq, France).

Chromatin immunoprecipitation

For ChIP, 1.5 × 10^6 monocytes were crosslinked in 1% formaldehyde (Mallinckrodt-Baker, Dublin, Ireland) for 10 min, treated with 1/10 volume of 1.25 M glycine (J.T.Baker, Thermo Fisher, Waltham, MA, USA) for 3 min, washed with ice-cold phosphate-buffered saline and stored in −80 °C after snap freezing. Crosslinked monocytes were lysed using 1% SDS and 20 mM HEPES, and sonicated using a Biorupter sonication device (Diagnode, Seraing, Belgium). Chromatin sonication (DNA fragments between 150–600 bp) was validated by agarose gel electrophoresis. Sonicated chromatin was divided into two fractions and used as either input material or for overnight H3K4me3 precipitation using protein G dynabeads (Life Technologies, Oslo, Norway) and polyclonal H3K4me3 antibody (Catalog number C15410003-010, Diagnode). DNA was isolated by de-crosslinking input DNA and H3K4me3 precipitated DNA in elution buffer (1% SDS, 0.1 M NaHCO3, 0.19 M NaCl, 2 units Prot K (Thermo Scientific)) at 65 °C for 4 h followed by phenol/chloroform DNA precipitation. Quantification of immunoprecipitated DNA was performed by quantitative PCR using primers provided in Supplementary Table S2. Primers were located in the promotor

regions of IL6, TNFα and RPL5 as shown by H3K4me3 containing regions in monocytes via publicly available ChIP-sequencing data obtained from ENCODE/Broad institute (GEO accession: GSM1003536). Insulin, which is not expressed by monocytes, was used as negative control.

RNA isolation, cDNA synthesis and expression analysis

RNA isolation and cDNA synthesis was performed as previously described.[29] Two housekeeping genes were used for normalization: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-2 microglobulin (*B2M*). Relative expression was calculated using the ΔΔCT method.[30] The sample with the lowest expression was set to 1. Primer sequences are listed in Supplementary Table S2. Before quantitative PCR, minus RT samples were checked for genomic DNA contamination.

Cytokine production

TNFα and IL6 levels in the medium of cultured monocytes were measured using human TNF ELISA kit (BD Bioscience, San Jose, CA, USA) or human IL6 DuoSet ELISA (R&D System, Abingdon, UK), respectively. TNFα and IL6 in serum were determined using the Milliplex Human Adipocyte panel (Millipore, Amsterdam, The Netherlands) and measured on the Bio-Plex array reader and Bio-Plex software in accordance with the manufacturer's instructions.

Statistical analysis

Data are presented as means±s.d. Statistical analysis was performed using GraphPad (GraphPad, San Diego, CA, USA). Mann–Whitney *U*-test was performed to determine significant differences. Differences with *P*-values <0.05 were considered statistically significant. Sample size calculation was performed based on the observed effect size in β-glucan-trained monocytes using G power v3.1 (University of Düsseldorf, Germany).

Supplementary information

Supplementary information is available online on the Genes and Immunity website (http://www.nature.com/gene): Supplementary Figure S1-S7 and Supplementary Table S1 and S2.

Acknowledgements

We are grateful for the RA patients and the healthy volunteers for donating blood. This work was supported by the Dutch Arthritis Foundation, The Netherlands. FK has support from UNESCO-L'Oreal for Women in Science Fellowship, Marie Curie FP7 Outgoing Fellowship and an LUMC Research Fellowship. The work of VdH-vM was supported by a Vidi-grant of the Dutch Organisation for Scientific Research.

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