Investigating the role of pathogenic factors in inflammatory joint disease

Ph.D thesis

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1. Introduction

The Th17 cells and osteoclasts have a prominent role in both rheumatoid and psoriatic arthritis. Th17 cells are a subpopulation of T helper lymphocytes which produce IL-17A and in human express *RORC* transcription factor. They play an important role in the immune defense against extracellular pathogens and in the pathogenesis of several autoimmune diseases. The increased differentiation and cytokine production of Th17 cells was described in chronic autoimmune inflammation, which may lead to altered cell function both in the joint (e.g synoviocytes and osteoclasts), or in the skin (keratinocytes). Osteoclasts are bone resorbing myeloid cells. Increased activity of osteoclast was observed in inflammatory joint diseases like rheumatoid (RA) and psoriatic arthritis (PsA).

Cigarette smoking is a major environmental risk factor of RA, smoking may regulate the differentiation and function of T cells via aryl hydrocarbon receptor (AHR). In addition, the immune complexes (IC) also play a

central role in the pathogenesis of RA, ICs are regulators of both osteoclast and T cell functions.

2. Objectives

2.1. To study the human *in vitro* Th17 differentiation in healthy donors.

2.2. To investigate the effect of cigarette smoke and the SICs on the *in vitro* Th17 and osteoclast differentiation.

2.3. To study the *in vitro* Th17 cell differentiation in RA and PsA.

3. Methods

Donors: patients were recruited in the Hospital of Hospitaller Brothers of St. John of God, Budapest, Hungary. They were diagnosed with RA ($n = 12$) according to the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria, or with PsA ($n = 12$). Matched control samples were collected from healthy volunteers ($n = 12$).

In vitro cell cultures:

Th17 differentiation: peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers, RA, and PsA patients by density gradient centrifugation over Histopaque-1077 (Sigma, Darmstadt, Germany). $CD4^+CD45RO^-$ T cells were negatively separated from PBMCs with a magnetic-activated cell sorting $CD4^+$ T Cell Isolation Kit and CD45RO Microbeads (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cells were cultured (10^6/mL) in RPMI 1640 (Sigma, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Sigma, Darmstadt, Germany), 1% L-glutamine, and 1% penicillin–streptomycin solution (Sigma, Darmstadt, Germany). The naive T cells were stimulated with anti-CD3 (1μg/ml; Bio-Techne Ltd., R&D systems, Abingdon, United Kingdom), anti-CD28 (1 μg/mL; BioLegend, Inc., San Diego, CA, USA), and with F(ab′)2 fragment goat anti-mouse IgG (CAB) (1 μg/mL; Jackson ImmunoResearch Inc., West Grove, PA, US) antibodies, and treated with TGFβ (2.5 ng/mL), IL-6 (25 ng/mL), IL-1β (10 ng/mL), and IL-23 (10 ng/mL) cytokines (all from

ImmunoTools GmbH, Friesoythe; Germany). Anti-IL4 antibody (10µg/ml, BioLegend, Inc., San Diego, CA, USA) was used during all cytokine treatments. The cells were treated for 10 days and samples were collected initially, on the 5th and 10th days for analysis.

Osteoclast differentiation: $CD14^+$ cells were separated from PBMCs using positive magnetic isolation method (EasySep, StemCell Technologies, Vancouver, Canada). Monocytes (10^5/well) were cultured in alpha-minimal essential medium (alpha-MEM) (Sigma, Darmstadt, Germany) with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1% L-glutamine (Sigma, Darmstadt, Germany) and 1% Penicillin–Streptomycin (Sigma, Darmstadt, Germany). The cells were stimulated with 50 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF) for 24 h (PeproTech, London, UK). Thereafter the samples were treated with both recombinant human receptor activator of nuclear factor kappa B (RANKL) and M-CSF (PeproTech, London, UK).

Human IgG (Jackson Immunoresearch, Baltimore Pike, PA, USA) and recombinant Staphylococcal Protein A

(rSPA) containing (Repligen, Waltham, MA, USA) soluble immune complexes (SIC) were applied in dilution 1:100 or 1:200 during osteoclast and Th17 cell differentiation. The differentiated cells were collected for gene expression analyses.

Preparation of tobacco smoke treated medium: sterile, FBS free RPMI (Sigma, Darmstadt, Germany) media was exposed to cigarette smoke. The treated media was diluted 25x and completed with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1-1% antibiotics/antimicotics solution (Sigma, Darmstadt, Germany)

Cell viability test: cell viability was evaluated by Trypan blue staining and by an impedance-based cell analyzer (CASY-TT, Roche Innovatis, Penzberg, Germany).

Gene expression analysis:

Th17 differentiation: total RNA was isolated with NucleoSpin RNA/Protein kit (MACHEREY-NAGEL GmbH & Co., Düren, Germany) and cDNA was synthesized from 25ng RNA/gene with a SensiFAST cDNA Synthesis Kit (BioLine Reagents Ltd, London,

UK). The quatitative real-time polymerase chain reactions (PCRs) were carried out in Master Mix containing SensiFAST™ Probe Hi-ROX Kit (BioLine Reagents Ltd, London, UK) using TaqMan assays (Thermo Fisher Scientific, Waltham, MA, USA) for *HPRT-1* or *RORC* or *TBX21* with ABI Prism 7900ht instrument.

Osteoclast differentiation: total RNA was prepared from OC cultures using an RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). cDNA was generated and PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System. Primers for the following genes were used: CALCR, C-FOS, CTSK, C-Fms, DC STAMP, NFATc1, OSCAR, RANK, TRAP and SLAP-1, 2 (IDT, San Jose, CA, USA). Specific transcript levels were normalized to those of GAPDH and then to control samples, and the ΔΔCt calculation method was used to determine gene expression.

Flow cytometry: the cells were stained with anti-human CD196 (CCR6) FITC, anti-human CD194 (CCR4) PE, anti-human CD183 (CXCR3) PerCP Cy5.5, anti-human CD3 APC Cy7, anti-human CD4 PerCP Cy5.5, anti-human CD45RA FITC, anti-human CD45RO PE Cy7, anti-human CD197 APC antibodies or with the appropriate isotype controls (all from BioLegend, Inc., San Diego, CA, US). After washing, cells were measured with fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). To determine the RORγ and TBET expression cells were permeabilized and fixed using transcription buffer set (BD Biosciences, San Jose, CA, USA) and stained with anti-human T-bet PE CF594, or with anti-human RORγ PE (both from BD Biosciences, San Jose, CA, USA) antibodies. Cells were measured with BD FACS Aria III. flow cytometer (BD Biosciences, San Jose, CA, USA), analysed with FlowJo (Tree Star, Ashland, OR, USA).

ELISA and ELISPOT measurements: IL-17A and IL-22 cytokine levels were measured with human IL-17A /homodimer/ ELISA Ready-SET-Go!™ Kit and IL-22

ELISA Ready-SET-Go!™ Kit (Fisher Scientific, Loughborough, UK). The IL-17A producing cell number was measured with human IL-17A ELISPOT Ready-SET-Go!™ IL-17A kit (Invitrogen™ eBioscience™, Fisher Scientific, Loughborough, UK).

Confocal microscopy: naive and memory T cells were fixed, permeabilized and Fc receptors of the cells were blocked by mouse sera and AHR was stained with 5µg/ml monoclonal anti-human AHR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and stained with 4µg/ml goat anti-mouse Alexa 488 antibody (Thermo Fisher Scientific, Waltham, MA, USA) and DRAQ5, the cells were analysed by FluoView 500 confocal laser scanning microscope.

Statistic: GraphPad Prism Software V6 (San Diego, CA, USA), the R statistical software and SPSS were used for statistical analyses.

4. Results

4.1. Study the human *in vitro* Th17 differentiation in healthy donors.

The *RORC* expression of T cells was increased by TGFβ+IL-6+IL-1β treatment after five days. The applied goat anti-mouse Fab fragment cross linking antibody increased the anti-CD3/CD28 induced T cell activation, reflected by the increased IL-2 production. We observed two populations of both PBMC and $CD4^+$ T cells (approximately in 50-50%) with different size range (7-9μM and 9-13μM), following activation.

The *RORC* expression of $CD4^+$ T cells was 4x higher compared to PBMCs upon TGFβ+IL-6+IL-1β treatment. IL-4 and IFNγ neutralizing antibodies contributed to the TGFβ+IL-6+IL-1β induced Th17-differentiation, based on the increased *RORC* expression and IL-17A production. Both IL-17A and IL-22 production of $CD4^+$ T cells were induced by activation (anti-CD3/CD28+ goat anti-mouse Fab fragment cross linking antibody treatment). The Th17 specific *RORC* expression was also induced by several different cytokine treatments. The

Th1 related *TBX21* expression was also increased by Th17 inducing cytokine treatments.

4.2. Investigate the effect of cigarette smoke and the SICs on the *in vitro* Th17 and osteoclast differentiation.

AHR gene expression was observed in untreated naive and memory T cells of healthy donors with quantitative real-time PCR. There was no difference between the AHR mRNA expression of naive and memory T cells. AHR protein expression of both naive and memory T cells was increased by anti-CD3/CD28+cross linking antibody induced activation, studied by confocal laser microscopy.

Anti-CD3 induced IL-17 production of PBMCs was decreased, while the cell viability did not change upon benzo[a]pyrene, TCCD treatment and cigarette smoke exposure, as measured by ELISPOT and flow cytometry methods. The AHR agonist leflunomide treated RA patients' T cells produced lower levels of IL-22 compared to the biological (Cimzia and Roactemra) therapy treated patients' samples ($p<0.01$). In addition,

the IL-22 production of leflunomide treated patients derived cells were more intensively increased by activation+TGFβ+IL-6+IL-1β+anti-IL4 than biology therapy treated patients ($p<0.01$).

The *TBX21* expression of cytokine (activation+TGFβ+ IL-6+IL-1β+anti-IL4) treated cells was decreased by SICs compared to the untreated, activated and only cytokine treated cells. Furthermore IL-17A (but not IL-22) was induced effectively by immune complexes.

The *CTSK* and *RANK* expression of healthy derived osteoclasts were inhibited by SICs, which was not observed neither in RA nor in PsA derived osteoclasts.

4.3. Study the *in vitro* Th17 cell differentiation in RA and PsA.

The healthy memory T cells expressed higher levels of *RORC* and *TBX21* compared to naive cells, which difference was not observed in case of RA or PsA derived samples. Strong linear correlation was observed between the *TBX21* ($r = 0.9$, $p <0.0001$) levels of naive and memory T cells in all three donor groups; by contrast

there was no correlation between the *RORC* levels (r = 0.18, p = 0.17) of naive and memory T cells.

The RA and PsA patient derived naive T cells are characterized by higher *RORC* gene and RORγ protein but not higher *TBX21* gene and TBET protein expression compared to healthy donors. We observed by flow cytometry a naive T cell population with increased RORγ levels in both patient groups. There was no difference in the *RORC* or *TBX21* gene expression of memory T cells between the different patient groups. The majority of memory cells from all groups were central (not effector) cells. The effector memory cells were more frequently $TBET^+$ or $ROR\gamma^+$ or $TBET^+ROR\gamma^+$ than the central memory cells. Unlike RA or PsA derived T cells, there were more $CCR6^+CCR4^+$ and $CCR6^+CXCR3^+$ expressing memory T cells of healthy donors, compared to naive cells. In addition, there was no difference in the Th1 associated CXCR3 expression of naive and memory cells in the donor groups.

The $CCR4^+$ cells in all patient groups correlated with the erythrocyte sedimentation rate (ESR; r=0.7213;

p=0.0093) and with the C-reactive protein (CRP; r=0.7743; p=0.0043) levels.

Linear discriminant analysis was performed to evaluate the data from naive and memory T cell-derived *RORC* and *TBX21* expression. The discriminative power of the healthy, RA, and PsA group separation was 61% and the naive T cells' *RORC* expression had the strongest determinant role.

Naive T cells from healthy individuals, RA, and PsA patients were discriminated from each other with 81.3% power by the chemokine receptor expression data and the determinant factor was the proportion of $CCR4^{+}CXCR3^{+}$ memory T cells. Healthy donors, RA, and PsA patients were discriminated from each other with 100% power if all parameter were analysed, i.e. more parameters provide better discrimination from healthy donors.

The activation +TGFβ+IL-6+IL-1β+anti-IL4 treatment increased *RORC* but not *TBX21* expression in all donor groups. *TBX21* expression was increased by IL-23 and IL-1β treatments, which was most evident in healthy donors. In healthy donors both transcription factors were

increased by activation, while only *RORC* expression was increased in PsA and none of them in RA.

The *TBX21* expression of PsA derived T cells did not change by any treatment applied. The TGFβ+IL1β +IL6+anti-IL4 and IL1β+IL23+anti-IL4 treatments (both $p<0.01$) in RA, while the TGFβ+IL6+anti-IL4 and TGFβ+IL1β+IL6+anti-IL4 treatments (both $p<0.05$) in PsA increased the *RORC* expression. All treatments, except the TGFβ+IL-6+anti-IL-4 altered the $CCR6^{+}CCR4^{+}$ cell ratio in healthy donors.

Both IL-17A and IL-22 production was increased by all cytokine treatments. Activation did not induce transcription factor expression, but stimulated cytokine production in both patient groups. TGFβ + IL6 + anti-IL4 antibody treatment decreased the IL-22 secretion of healthy- and PsA-derived but not those of the RA-derived T cells ($p < 0.05$ and $p < 0.05$, respectively) and the IL-17A production of the PsA T cells ($p < 0.01$).

Unlike the IL-17A and IL-22 cytokine production, compared to the 5 days data, there was no additional difference in transcription factor expression at the 10^{th} day.

In addition, only in patients the $CCR6^{+}CCR4^{+}$ cell ratio was increased by IL-1β+IL-23+IL6+anit-IL4 treatment. Healthy donors, RA, and PsA patients were discriminated from each other with 81%, according to the cytokine production, in which the determinant factor was the IL1β+IL23+IL-6+anti-IL4 treatment induced IL-22 production. The IL-17A production pattern was altered in healthy donors compared to both RA ($p = 0.0000026$) and PsA ($p = 0.0001$) patient groups. Unlike IL-17A, the IL-22 production pattern was similar in PsA and healthy ($p = 0.58$), while different in healthy compared to RA ($p = 0.000006$) or RA compared to PsA ($p = 0.001$).

4. Conclusions

4.1. The human *in vitro* Th17 differentiation was optimally induced from $CD4^{+}CD45RO^{-}$ naive T cells. The anti-CD3, anti-CD28 and cross-linking antibody further induced the T cell activation; the IL-4 and IFNγ neutralizing antibody treatment contributes to the differentiation.

4.2. AHR may have a role in the signalization induced by environmental pollutants (e.g. cigarette smoke components TCDD or B[a]P) or drug molecules (e.g. leflunomide). The *AHR* expression of human naive and memory T cells was induced by activation.

TCDD, benzo[a]pyrene and leflunomide AHR agonist ligands have different effect on IL-17A and IL-22 production of lymphocytes.

TBX21 expression was inhibited; on the contrary the IL-17A production was increased by SICs during Th17 cell differentiation. *CTSK* and *RANK* expression was also inhibited by SICs in healthy donors, but not in RA or PsA.

4.3. RA and PsA derived naive $CD4^+$ T cells show an early commitment towards the Th17 lineage. The *in vitro* Th17 differentiation is differently regulated in RA and PsA.

5. Bibliography

5.1. Publications relevant to the dissertation:

- **E. Baricza**, N. Marton, P. Királyhidi, O.T. Kovács, Székely I. Kovácsné, E. Lajkó, L. Kőhidai, B. Rojkovich, B. Érsek, E. Buzás, G. Nagy, (2018) *Distinct in vitro Th17 differentiation capacity of peripheral naive T cells in rheumatoid and psoriatic arthritis.* FRONTIERS IN IMMUNOLOGY 9:(Apr) Paper 606. 13 p. (2018)

  IF: 5,511

- **E. Baricza**, V. Tamási, N. Marton, E. I. Buzás, G. Nagy (2016) *The emerging role of aryl hydrocarbon receptor in the activation and differentiation of Th17 cells.* CELLULAR AND MOLECULAR LIFE SCIENCES 73:(1) pp. 95-117. (2016)

  IF: 5,788 (review article, not included in cumulated IF)

- N. Marton, O.T. Kovács, **E. Baricza**, Á. Kittel, D. Győri, A. Mócsai, FMP Meier, CS Goodyear, IB McInnes, E. I. Buzás, G. Nagy (2017) *Extracellular vesicles regulate the human osteoclastogenesis:*

divergent roles in discrete inflammatory arthropathies. Cell Mol Life Sci, 74:3599-3611.

IF: 6,721

5.2. Other publications:

- N. Marton, E. **Baricza**, B. Érsek, E. I. Buzás, G. Nagy (2015) *The Emerging and Diverse Roles of Src-Like Adaptor Proteins in Health and Disease.* Mediators Inflamm, 2015: 952536.

  IF: 3,418 (review article, not included in cumulated IF)

- V. Csöngei, L. Járomi, E. Sáfrány, C. Sipeky, L. Magyari, N. Polgár, J. Bene, P. Sarlós, L. Lakner, E. **Baricza**, M. Szabó, G. Rappai, B. Melegh (2011) *Interaction between CTLA4 gene and IBD5 locus in Hungarian Crohn's disease patients.*

  INTERNATIONAL JOURNAL OF COLORECTAL DISEASE 26:(9) pp. 1119-1125.

  IF: 2,385

- M. Mohás, P. Kisfali, E. **Baricza**, A. Mérei, A. Maász, J. Cseh, E. Mikolás, I. A. Szijártó, B. Melegh, I. Wittmann, (2010) *A Polymorphism within the Fructosamine-3-kinase Gene is Associated with HbA1c Levels and the Onset of Type 2 Diabetes*

Mellitus. EXPERIMENTAL AND CLINICAL ENDOCRINOLOGY & DIABETES 118: (3) pp. 209-212.

IF: 1,826

Cumulative impact factor: **16,443**