

Network: Infection and Inflammation: from Pathogen-induced Signatures to Therapeutic Target Genes

Project: Clinical Validation by Custom cDNA Array

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Introduction

In Rheumatoid arthritis (RA), not only synovial tissue macrophages (M ϕ) but also peripheral blood Monocytes (MO) are activated. Upon activation both cell types spontaneously release inflammatory cytokines and mediators, which in turn cause and sustain joint inflammation and destruction of connective tissue. Up to now, genes and pathways involved in RA monocyte activation are only partially characterized.

In particular DNA microarrays are used to identify genes differentially regulated in RA. So far, these analyses identified: i.) RA disease relevant genes, ii.) MO/M ϕ activation patterns in RA and in other chronic inflammatory diseases, iii.) Differentially regulated genes in anti-TNF α treatment with prognostic value, iv.) Pathways activated or blocked during different stages of the disease, and additionally v.) Potential therapeutic intervention sites. Based on these results a custom cDNA microarray was designed in NGFN-1. This array will be used within NGFN-2 for analysing RA patient MO blood samples for diagnosis, the detection of individual RA subtypes, and the responses of patients to different types of treatment. In parallel, the custom MO microarray will be expanded by genes altered in expression in synovial M ϕ and T-cells isolated from RA patients.

Results/Project Status

Rheumatoid arthritis (RA) is a systemic inflammatory disease leading to joint destruction and ultimately loss of function. Especially cells of the monocyte/macrophage (MO/M ϕ) system play a key role in mediating the course of RA. MO/M ϕ signature genes have been identified to be suitable for diagnosing the disease, its progression, and the success of treatment with TNF α (Adalimumab). Additionally, MO/M ϕ signature genes were found to be involved not only in chronic inflammatory diseases such as RA but also in other chronic inflammatory diseases, in cancer or in infectious diseases.

Custom MO microarray and preselection of candidate genes for RA and therapy outcome

These probes were identified in differential hybridisation by gene subtraction of RA-MO versus normals. A further source of gene probes was whole genome analysis (U133A/B) of MO in samples from normals, RA patients prior to and during anti-TNF α treatment (n=7 each; Fig.1 and Fig.2).

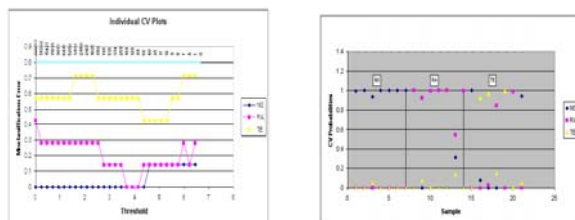


Fig. 1: Application of PAM to identify genes determining response to anti-TNF α therapy. A.) A threshold of 4.5 was applied.

B) Probabilities for correct and mis-classification were calculated and identified treated patients TNF-4 and TNF-6 as RA-group. (ND = normal donor; TE = treated RA patient).

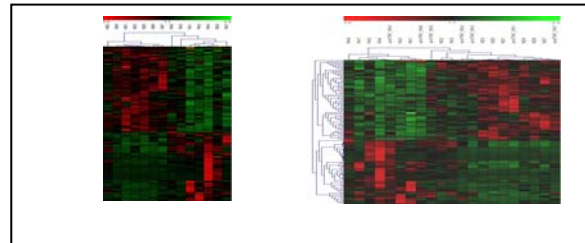


Fig. 2: Hierarchical Clustering and molecular measures for response to anti-TNF α treatment. A.) Clustering of RA versus normal donor MO and B.) of RA versus ND and versus anti-TNF α treated patients.

Using this gene selection we designed a custom cDNA microarray. The unique collection of gene probes and the established SOPs for array fabrication, RNA isolation, cDNA synthesis and hybridisation provide the basis for the production of robust medically relevant data.

Recently, a custom RA-MO microarray was established in close collaboration with the Kernbereich Plattform-2 (Max-Planck-Institute for Molecular Genetics; Lehrach, Hultschig; Berlin). This customised array contains RA relevant gene probes immobilised as denatured sequence verified PCR products (Fig.3). The successful design of the customised RA microarray for the detection of MO activation was verified by comparative analysis of non-stimulated and stimulated (LPS, PMA, Vit.D3+LPS, PMA+LPS) premonocytic U937 cells, and of non-stimulated and stimulated healthy donor MO. In addition, gene expression profiles of MO from RA patients prior to and during TNF α treatment were determined.

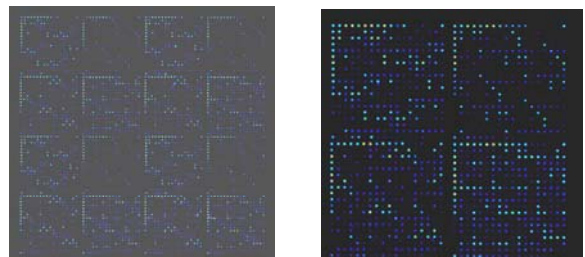


Fig.3: RA-MO-Chipset-II — Complex hybridization A) 4x4 fields (c) were spotted, each containing 18*19 spots, including positive and negative controls. Each field contains the same set of genes and controls. **B)** Four types of subfields were produced with different arrangement of the spots to identify variability due to the spotting procedure.

In part, these patient samples and normals have been already analysed using Affymetrix U133A/B microarrays. All results obtained with the customised microarrays are in excellent agreement with previous analyses of normal and patient MO samples, and U937 cells. The gene selection of the candidate genes to diagnose RA disease and furthermore to calculate the response of anti-TNF α treatment will be verified in close cooperation with the TP48 (Grützka) and with the pharmaceutical industry partner Abbott. In this clinical study we will use a RNA probe set from patients prior to treatment and during therapy at 3 different time point (4weeks, 12w and 26w) from 30 patients and 15 healthy donors. Using this probe set too, genome

wide array results were verified by Real-Time TaqMan polymerase chain reaction with 20 candidate genes. So far, we could demonstrate that this candidate gene selection allow to define disease activity and furthermore allow to define the individual patient response during therapy with anti-TNF α . Good correlations and high statistical significance with between gene expression and clinical data were obtained.

So far our custom RA-MO cDNA microarray includes RA-MO specific up and down regulated genes (RA versus normals) and genes, which are differentially expressed in MO from healthy controls, RA prior to and during anti-TNF α treatment. The array is divided into 4 blocks containing a probe set of 311 cDNAs. The RA-MO microarray is designed so that 16 replicates of each cDNA probe are spotted alongside with positive cDNA samples (housekeeping genes and commercial spike controls; Stratagene), negative controls, and guide dots. In our approach we rely on well. The unique collection of gene probes and the established SOPs for array fabrication, RNA isolation, cDNA synthesis and hybridisation provide the basis for the production of robust medically relevant data.

Currently the customized RA-MO-Chipset is used as reference in establishing a novel protocol for accelerated and financially attractive amplification and labelling in co-operation with Amersham-Pharmacia and Services in Molecular Biology (SMB; GbR), a small medium enterprise (SME). This novel amplification protocol will be of high interest in high-throughput screening of many patients in particular with respect to the limited amounts of blood or tissue (50ng total RNA). It will be provided to other NGFN-2 partners. Codelink slides (Amersham) were tested as an alternative carrier for DNA probes. With this approach we anticipate an improved comparability of the results obtained on different customised and commercial array platforms in laboratories of the NGFN network.

Bioinformatic gene expression analysis were performed in close co-operation with the MPI-MG (R.Herwig), the "Labor für Genomforschung an der Charité" (R.Kuban) and the SME Oligene GmbH (J.Grün), a Charité spin-off.

Functional analyses of candidate genes

Furthermore, a novel differentially regulated gene was identified and characterized. The full length transcript was cloned by 3'- and 5'RACE strategies and 11 different splicing variants and one natural anti-sense RNA acting as regulator transcript *in cis* and encoded from the opposite DNA strand were cloned, too. siRNA knockdowns experiments were to define gene regulation using genome wide Affymetrix and Codelink microarrays. The knockdown regulate genes which are in close relation to ubiquitination processes. The hypothetical translated protein bears a 35AA long domain with 100% homology to recently described E2 ubiquitinases of the RNF5 family. The protein terminus is a transmembranic AA composition. This is be due to our microarray results of gene silencing. Therefore, also a lot of

proteins which are located in the plasma membrane were differentially regulated.

Outlook

Microarrays are important, novel tools for characterization of disease activity and response to anti-TNF α therapy. They can be applied as screening systems for **1)** diagnosis of arthritis, **2)** therapeutic effectiveness, **3)** investigation of drug effects, and **4)** target identification for new therapeutics (anti-CD20; TP48; Grützkau). Thus, the experimentally defined current selection of genes on our cDNA microarray (RA-MO chip-set-II) could also contribute to the investigation of the role of MO in other rheumatic diseases and therapy studies, and to improve the understanding of regulated MO pathways. The RA-MO chipset-II is cost-effective, competitive, flexible, and applicable for different MO/M ϕ -oriented questions in RA, infectious disease, or other states of chronic inflammation. The customized microarray will be added by other candidate genes from synovial tissue (Häupl, Berek) and also from MO (TP48, Grützkau). Furthermore, several other functionally unknown candidate genes will be characterized by siRNA knockdown strategies. The will be to investigate their pathway regulation and function in chronic diseases or cancer. Several patents national and international patents were submitted. Cooperations with the pharmaceutical industry might open new avenues to define novel drugs for effective treatment and hopefully will force the national economics.

Lit.: 1. Dorffel Y., Bresan V., Stuhlmüller B., Dorffel W.V., Pruss A., Scholze J.: Candesartan – an inhibitor of monocyte activation in hypertensive patients. Perfusion 2002; 15: 96-101 (2002). 2. Scott K., Bernier F., Stuhlmüller B.: Ring up the curtain on DING proteins. EMBO Lett. 2002 524: 6-10. 3. Häupl T., Burmester G.R., Stuhlmüller B.: New aspects of molecular biology diagnosis. Array technology and expression profile for characterization of rheumatic diseases. Z. Rheumatol. 2002; 61: 396-404. 4. Stuhlmüller B., Kunisch E., Franz J., Martinez-Gamboa L., Hernandez M., Pruss A., Ulbrich N., Erdmann V.A., Burmester G.R., Kinne R.W.: Detection of oncofetal H19 RNA in rheumatoid arthritis synovial tissue. Am J Pathol. 2003; 163: 901-911. 5. Häupl T., Krenn V., Stuhlmüller B., Radbruch A., Burmester G.R.: Perspectives and limitations of gene expression profiling in rheumatology: New Molecular Strategies. Arthritis Rheum. 6: 131-137 (2004) 6. Müller R., Skapenko A., Grunke M., Wandler J., Stuhlmüller B., Kalden J.R., Schulze-Koops H.: Regulation of myeloid cell function and MHC class II expression by TNF. Arthritis Rheum. 2005; 52: 451-460. 6. Kinne R.W., Stuhlmüller B., Palombo-Kinne E., Burmester G.R.: Rheumatoid Arthritis: Section Mechanisms of Inflammation. Chapter: The role of macrophages in rheumatoid arthritis The New Frontiers in Pathogenesis and Treatment; Eds. G. Firestein, G. Panayi, F. Wollheim).