General Discussion

Partly adapted from:

Verweij CL, Vosslamber S.
The type I interferon system in rheumatoid arthritis.
Nova Science Publishers, Inc. 2013. Book chapter

Verweij CL, Vosslamber S.
The type I interferon system in multiple sclerosis: towards a personalized medicine approach for interferon-β therapy.
Nova Science Publishers, Inc. 2013. Book chapter

Verweij CL, Vosslamber S.
Relevance of the type i interferon signature in Multiple sclerosis towards a personalized medicine approach for interferon-beta therapy.
Discov med. 2013 jan;15(80):51-60. Review

Verweij CL, Vosslamber S.
New insight in the mechanism of action of rituximab: The interferon signature towards personalized medicine.
Discov med. 2011 Sep;12(64):229-36. Review

Vosslamber S, van Baarsen LG, Verweij CL.
Pharmacogenomics of IFN-beta in Multiple sclerosis: towards a personalized medicine approach.
Pharmacogenomics. 2009 jan;10(1):97-108. Review

SCOPE OF THE THESIS

MS and RA are complex diseases that are heterogeneous in nature. Variability in drug-responsiveness exemplifies this heterogeneity and suggests that for both MS and RA, underlying disease mechanisms may vary among patients. It appeared that differential activity of the type I IFN pathway is present in a subset of patients with several autoimmune diseases and is characteristics for the disease heterogeneity. Here we set-out to study the role of IFN activity in relation to IFNβ therapy in MS and B-cell depletion therapy via rituximab in RA. Besides the relation of IFN activity to treatment outcome, we also studied the role of type I IFN activity in autoimmunity in relation to pathogenic or clinical characteristics. In RA we studied the relation of *IRF5* genetics to cardiovascular disease, since it is well known that IFNβ is an important mediator in this process. In patients with Inflammatory Idiopathic Myopathies, the relation between autoantibodies that play an important role in this disease, and type I IFN activity was studied. Increased understanding of the molecular base of drug responsiveness will ultimately enable improved treatment strategies and personalized medicine in these diseases.

MOLECULAR MARKERS FOR IFNß TREATMENT IN MS

Nearly every aspect of a disease phenotype is represented by pathophysiological processes driven by genes and their products. These represent a molecular signature that is associated with disease characteristics and thus defines the samples unique biology. Genomics technology enabled us to provide sufficient knowledge to determine these processes in relation to disease heterogeneity and possibly treatment outcome.

PHARMACODYNAMICS OF IFNß TREATMENT IN MS

Large scale molecular profiling technologies are widely applied to identify pharmacodynamic changes for IFNβ in healthy controls and MS patients. However, whereas these studies provided insight into the IFNβ responsiveness in terms of a group average the issue of inter-individual heterogeneity was not addressed extensively and, as a consequence, they did not provide markers to predict treatment outcome. Since a high proportion of about 40% of the patients do not or only poorly respond to IFNβ and non-responsiveness can only partly be explained by immunogenicity, i.e. the development of neutralizing antibodies, it is likely that other mechanisms that result in insensitivity and/or resistance to the effects of IFNβ underlie differential responsiveness. This implies that baseline characteristics and/or pharmacodynamic responses may differ among patients, leading to inter-individual differences in clinical efficacy.

In chapter 1.1 we aimed to unravel the heterogeneous baseline characteristics that relate to, and might be predictive for, treatment outcome. We studied genome wide transcription profiles of MS patients starting with IFNβ treatment and showed that increased activity of the type I IFN system, reflected by a baseline IFN type I signature, determined the pharmacological response to IFNβ. Given the beneficial role of IFNβ on disease activity in RRMS these results appear counterintuitive. Moreover, this increased IFN-activity at baseline was associated with an absent pharmacological response to IFNβ-treatment in RRMS. This absence of a pharmacological response in the MS patients with a high type I IFN status at baseline was consistently observed over time, at one, three and six months after the initiation of the therapy and was confirmed in an independent cohort of 30 RRMS patients. One possible explanation for these observations is that the type I IFN activity in patients with this type I IFN signature was already saturated prior to the initiation of the therapy, resulting in the absence of a pharmacological effect of administrated IFNβ in these patients. Indeed, we demonstrated that PBMC of RRMS patients with increased baseline type I IFN activity had lost the capacity to respond to *in vitro* stimulation with IFNβ, consistent with the *in vivo* findings. These data suggested that the *in vivo* pharmacological response to IFNβ treatment is dependent on the intrinsic differences in the blood cells reflected by an activated type I IFN activity prior to the initiation of the therapy. This was further confirmed by a study of Comabella et al[1] who not only validated the increased type I IFN activity at baseline in a subset of RRMS patients but also showed that baseline phospho-STAT1 levels were significantly higher in monocytes of those patients compared to those without an activated type I IFN system. Upon stimulation of the monocytes with IFNβ no differences between the phospho-STAT1 levels were observed.[1] These findings are consistent with our results, which revealed that in the patients with a baseline type I IFN signature, the type I IFN pathway is fully activated prior to therapy and cannot be activated further.

Our study set up was not sufficient to link clinical response to the pharmacological response or baseline type I IFN signature, however, Comabella et al[1] showed that type I IFN response genes were indeed predictive for the clinical outcome of IFNβ treatment in RRMS. In agreement with the results from our pharmacological outcome study, non-responders were indeed characterized by an increased expression of type I IFN response genes at baseline. The predictive accuracy of a gene set, consisting of predominantly type I IFN genes, reached 78%. These findings were replicated in an independent group of RRMS patients (predictive accuracy 63%). The genes that were selectively induced by type I IFN were found to be the best predictors of efficacy.

Several mechanisms could account for differential responsiveness to IFNβ treatment in MS patients. Although type I IFNs have an essential function in mediating innate immune responses against viruses, they may already be produced at very low levels[2] in a subset of MS patients. The presence of endogenous IFN might trigger negative regulators to inhibit

(further) activation of the JAK/STAT pathway. Accordingly, the interleukin-1 receptor-associated kinase 3 (IRAK3), a negative regulator of TLR4 signaling primarily expressed in monocytes was signficantly increased in IFN^{high} patients.[3] Since e.g. IFNα is known to desensitize further responses to IFNs[4], the presence of low endogenous IFNs could block further TLR and/or IFNβ induced signal.[5] Evidence exists that crosstalk with other cytokine-activated pathways such as the TNF pathway, could cause tachyphylaxis to type I IFNs.[6] The observation that the expression levels of negative type I IFN regulators such as SOCS1, SOCS3 and PIAS1 were similar between IFN^{high} and IFN^{low} RRMS patients[1], suggests that the interinvidual differences in IFN-activity are not explained by variation in the activity of inhibitory transcription factors.

A possible trigger for the elevated type I IFN activity remains unclear. Despite the capacity of blood cells to produce type I IFNs, proof for blood cells as the source for the type I IFN bioactivity is weak. Not surprisingly, there is no data showing increased levels of IFNα or IFNβ mRNA in peripheral blood cells of RRMS patients compared to healthy control. Support for the origin of type I IFN outside the peripheral blood compartment comes from the observation that both IFNα and IFNβ are present in brain tissue of patients with RRMS.[7,8] In acute lesions, IFNα and IFNβ were detected in endothelial cells, whereas macrophages expressed more IFNα, and IF-β was found in astrocytes. However, the simultaneous overexpression of genes of a common pathogen response pathway, including TLR signalling[9], is supportive for a possible role of TLRs in driving the innate immune response in RRMS.[1] Bacterial pathogens that activate the innate response via TLR2 and TLR4 generate a dominant NF-kB pathway, whereas viruses trigger the innate immune system through TLR3, TLR7 and TLR9 that are more involved in the activation of type I IFN production via the IRF3/7 pathway.[9] Comparative analysis of both response pathways with the gene signatures observed in the peripheral blood of RRMS patients revealed no significant activation of the NF-kB response pathway.[10] This observation argued against involvement of TLR2 and TLR4 in the innate immune activation in RRMS. The same study also reports that the pattern of gene expression profile of the IFN^{high} RRMS patients has a remarkable similarity with that of smallpox virus infected macaques, suggestive of a viral or virus-like stimulation. However, initial analyses on an association with detectable levels of viral DNA in PBMC and IgM or IgG seropositivity for EBV or HHV-6, two candidate viruses, were negative[1]. Although infectious agents such as viruses have long been considered as possible triggers for autoimmune responses in MS, a contribution for endogeneous factors, such as single- or double stranded DNA or RNA, in the activation of the innate response via TLR3, TLR7 or TLR9 cannot be excluded. For example, in SLE, it has been demonstrated that immune complexes between autoantibodies and nucleic acids released from necrotic or apoptotic cells can induce plasmacytoid dendritic cells to produce IFNα.[11] Thus the available data for RRMS do not exclude a role for endogeneous factors in the activation of the innate response in RRMS.

Not only the baseline type I IFN signature, but also significantly elevated serum concentration of IFNβ in non-responders compared to responders were reported.[12] High IFNβ concentrations correlated with increased IL-17F in the serum suggesting a tight biological association between these two cytokines. One possible explanation for the observation is that non-responders have aggressive T_H17 disease reflected by the in increase in IL-17F production, and IFNβ was produced to counteract inflammation. Alternatively, endogeneous IFNβ is pro-inflammatory during T_H17 disease. In that situation IFNβ treatment would not only be ineffective, but could also worsen symptoms as was shown in a Th17 induced experimental RRMS model.[12] However, the role of IL17F as predictor of the response to IFNβ could not be confirmed in an independent cohort.[13]

Altogether, these results warrant further studies to validate the clinical utility of the type I IFN signature as biomarker to predict the response to IFNβ treatment in RRMS.

ROLE OF *IRF5* GENETICS IN THE REGULATION OF TYPE I IFN ACTIVITY IN MS

Several genetic variants are known to contribute to the differential activity of the type I IFN system and one of the most described is the gene encoding *Interferon Regulatory Factor 5 (IRF5)*, a master regulator of the IFN/TLR pathway. *IRF5* is a transcription factor that functions as a central mediator of Toll-like receptor signaling and is involved in the production of type I IFN, apoptosis, cell-cycle regulation, cell adhesion and pro-inflammatory reactions.[14,15] Moreover, expression of *IRF5* is induced after activation of the IFN type I receptor, indicative that IRF5 is not only important in the production of type I IFN, but also in the regulation IFN type-I-induced gene activity.[16] Genetic variation in the *IRF5* gene has been found to be strongly associated with SLE, a disease wherein type I IFNs are clearly associated with disease activity and severity, and IFN response gene activity.[17-19]

In chapter 1.2 we studied the relation between *IRF5* genetics and response to IFNβ in MS patients. We found an association between genetic variation in *IRF5* and clinical and pharmalogical response to IFNβ treatment. Patients with the *IRF5* rs2004640-TT and rs47281420-AA genotype exerted a poor pharmacological response to IFNβ compared with patients carrying the respective G-alleles (P=0.0006 and P=0.0023, respectively). Moreover, patients with the rs2004640-TT genotype developed more magnetic resonance imaging (MRI)-based T2 lesions during IFNβ treatment (P=0.003). Accordingly, an association between MRI-based non-responder status and rs2004640-TT genotype was observed (P=0.010). For the rs4728142 AA-genotype there tends to be an association with more T2 lesions during IFNβ treatment and MRI-based non-responder status; however, this was not significant (P=0.103 and P=0.154, respectively). The clinical relevance of the rs2004640-TT genotype was validated in an independent cohort wherein a shorter time to first relapse was found (P=0.037).

The functional relevance of genetic variants of the *IRF5* gene might relate to it's influence on type I IFN levels since *IRF5* is a master regulator of type I IFN-activity and functions as a transcription factor when phosphorylated, leading to expression of downstream interferon response genes as well as production of type I IFN itself.[20] The rs2004640 *IRF5* SNP is located 2bp near the intron-exon boundary for exon 1b and creates an exon donor splice site which enables transcription of exon 1b. Splicing of IRF5 is highly complex and multiple *IRF5* isoforms are initiated at each promoter. Different isoforms can contain either exon 1a, 1b or 1c, depending on the promoter where transcription is initiated. Gene variants bearing exon 1a or 1b are constitutively expressed in pDCs and B-cells, whereas variants bearing exon 1c are inducible by type I interferons. The *IRF5* rs2004640 T allele, the allele that enables transcription of exon 1b, is associated with higher mRNA levels of IRF5.[21] Furthermore, it is known that the IRF5 isoforms differ in their ability to transactivate type I IFN genes, e.g. IFNα or IFNβ.[22] Thus the IRF5 rs2004640 T-allele is likely to enhance the expression of *IRF5* and successively type I IFN, including IFNβ, whereas the G-allele is not. The rs4728142 IRF5 SNP is located in the promoter region of *IRF5*. *IRF5* mRNA levels were also shown to be affected by rs4728142 genotype, suggesting a functional role of this polymorphism.

The exact pathophysiological role of IRF5 gene variants in relation treatment success in MS needs to be clarified but these findings suggest a role for IRF5 gene variation in the pharmacological and clinical outcome of IFNβ therapy and might have relevance as biomarker to predict the response to IFNβ in RRMS.

MOLECULAR MARKERS FOR RITUXIMAB TREATMENT IN RA

Although it has been proven that rituximab is effective in the treatment of RA, a substantial percentage of patients do not respond to treatment. Since rituximab equally depletes circulating B cells in responders as well as non-responders and many patients experience a relapse after 6 months when the B cell number is still low, the mechanism by which the clinical response is achieved is not entirely clear. It is speculated that more subtle B cell-related processes and/or indirect effects contribute to clinical benefit.

PREDICTION OF TREATMENT OUTCOME OF RITUXIMAB IN RA

Several studies proposed pathophysiological differences between responders and non-responders that could provide a basis for the identification of biomarkers to predict response to rituximab. Clinical parameters such as baseline disability, number of previously used TNF blocking agents, seropositivity as well as the reason for ineffectiveness of anti-TNF treatment are claimed to relate to treatment outcome.[23] But also variation in peripheral B-cell depletion,[24] differences in (long term) depletion and/or repopulation of

$IgD^+/CD27^+$ memory B cells [24-27,68] were associated with clinical outcome. These findings suggest that control of adaptive immune processes involving germinal center-derived, antigen-, and T-cell-dependently matured B cells is essential for successful RTX treatment. Other potential predictors of clinical outcome of rituximab treatment are serum levels of monocyte chemoattractant protein-1 and epidermal growth factor, which were found to be significantly higher after treatment in non-responders, as well as interleukine-6 genotype. Enhanced serum BAFF/BLyS levels and expression of BAFF receptor on naive and memory B cells, Fc-RIII genotype, blood cell transcripts and presence of Epstein-Barr virus genome in bone marrow are described to be positive predictors for clinical outcome. [28-33] Although the above findings have potential to contribute to a detailed insight in the mechanism of action related to efficacy and provide a framework to select biomarkers, clinical utility was not demonstrated.

With respect to response prediction, we showed in chapter 2.1 that the response to rituximab is associated with the activity of the type I IFN system prior to the start of treatment, reflected by differences if expression levels of a selective group of genes that have in common that they are all regulated by type I IFN. Patients that were identified as good responders based on their DAS at 6 months after start of treatment had a low or absent levels of at baseline of those type I IFN regulated genes, whereas non-responders displayed an enhanced expression of these genes already before the start of treatment. This association between baseline type I IFN activity and clinical response is in line with previous findings wherein it was demonstrated in two different cohorts (n=20 and n=31) that patients with a low IFN signature had a significantly greater reduction in the DAS28 and more often achieved a EULAR response at weeks 12 and 24.[34] In chapter 2.2 we validated the clinical utility of this IFN signature to predict non-response in an independent study using Receiver Operating Characteristic (ROC)-curve analysis whit the area under the (AUC)-curve as an important measure for test accuracy. Using advanced data-analysis, we identified a subset of 3 interferon response genes (IRGs) that most accurately and robustly predicts the response to rituximab therapy (AUC 0.87), which means that this test correctly classifies 87% of two patients of randomly drawn pairs, which is considered close to "excellent" (AUC>0.90).[35] Based on these data a cut-off could be chosen to predict non-response to rituximab with a specificity of 100% and a sensitivity of 44%. The association of IRG and RTX outcome appeared to be independent of other proclaimed biomarkers, among which seropositivity, number of previous anti-TNF blockers and baseline disability, for RTX treatment.

PHARMACODYNAMICS OF RITUXIMAB TREATMENT IN RA

Our genome-wide gene expression study as described in chapter 2.1 showed marked differences in the pharmacological response between patients. These differences relate to genes that represent several distinct biological processes, such as IFN-response gene

activity, humoral immunity, cytotoxic T and NK-cell immunity and chemotaxis. However, when patients were stratified based on their ΔDAS28 response at 6 months it became apparent that the only distinction between responders (ΔDAS28>1.2) and non-responders (ΔDAS28<1.2) was observed for pharmacological changes in the expression of type I IFN related genes. An increased expression of a set of 6 most discriminatory IFN response genes (*RSAD2, IFI44, IFI44L, HERC5, LY6E* and *Mx1)* at 3 months following single treatment is associated with a good clinical response, whereas the IFN-response activity did not change or slightly decreased in the non-responders. This applied also for the EULAR response criteria. After 6 months the IFN-response activity returned to baseline values, after which many rituximab treated RA patients experience a relapse of their clinical disease.

Results from these studies suggest that IFN^{high} RA patients represent a different pathogenic subset of RA marked by a failure to respond to B-cell depletion therapy. A simple explanation could be that the pathogenesis in IFN^{high} patients is less dependent on B-cells, compared to IFN^{low} patients. However, the IFN signature was found to be equally present in seropositive and seronegative RA patients, arguing against an association between IFN-response activity and pathogenic B cells.[36] Alternatively, a high baseline IFN-activity may be associated with the presence of a subset of pathogenic B cells insensitive to the effects of rituximab. These could be present at baseline and could survive in synovial or bone marrow tissues due to e.g. incomplete B-cell depletion or concomitant expression of B-cell survival factors such as BAFF/BLyS.[37] IFNs may also affect B-cell differentiation, such as in-situ differentiation in CD20-negative plasma blasts.[37] Another and more likely explanation would be that the evolution of the dynamic increase in IFN-activity following rituximab treatment is mechanistically more relevant to explain the difference between responders and non-responders. Thus the pharmacological induction of type I IFN-activity appears an important factor in the ameliorative effect of B-cell depletion therapy in RA and might relate to the increased BAFF/BLyS levels and persistence of pathogenic B cells.

A beneficial role for type I IFN in RA is highlighted by Treschow et al.[38], who showed that IFNβ-deficiency prolonged experimental arthritis. Subsequent transfer of IFN-expressing synoviocytes was beneficial in IFNβ-deficient recipients. Moreover, De Hooge et al.[39] demonstrated that deficiency of Signal Transducer and Activator of Transcription-1 (STAT-1), a crucial IFN induced signal transducer, resulted in exacerbation of experimental arthritis. However, despite these promising results in experimental arthritis, treatment of RA patients with IFNβ by itself was disappointing, which may be due to issues with dosing and pharmacokinetics.[40] Hence, an increase in IFN-response activity with concomitant B-cell depletion may be a prerequisite for a beneficial outcome. This hypothesis may also explain the beneficial effects of rituximab treatment observed in multiple sclerosis, a disease that responds beneficial to effects of IFNβ. Conversely, in IFN-type I driven diseases such as systemic lupus erythematosus (SLE) a pharmacological increase in the type I IFN activity

by rituximab may lead to disease progression and/or an increase in disease activity, and may explain the failure to meet clinical endpoints in recent randomized, placebo-controlled trials of rituximab.[41] This implies that rituximab might be less effective in those SLE patients who experience an increase in their type I IFN response activity levels during rituximab treatment.

TYPE I IFN SYSTEM IN RELATION TO DISEASE PARAMETER

IRF5 GENETICS AND CARDIAVASCULAR DISEASE IN RA

The association between *IRF5* genetics and gene expression based type I IFN signature in SLE and MS, inspired us to use it as a surrogate marker to study type I IFN activation in a patient cohort for which only DNA, and no RNA, samples were available. In RA, an increased cardiovascular morbidity and mortality is observed which cannot be fully explained by traditional CV risk factors. This makes it relevant to search for additional mechanisms linking RA to CV disease. The inflammatory processes that are ongoing in RA patients appear to be important in the increased cardiovascular morbidity and mortality although the exact mechanism remains unknown. This increased inflammatory state in RA (presented by increased immune cell activation, overproduction of inflammatory cytokines and increased CRP levels) together with the reduced presence of circulating endothelial progenitor cells (which can repair endothelial damage) lead to vascular injury.[42] In response to the vascular injury, vascular smooth muscle cell proliferation and intimal hyperplasia will be induced. Inflammatory cytokines contribute to atherosclerotic processes and Interferons are known inhibitors of proliferation. IFNβ is known to reduce the proliferation of vascular smooth muscle cells and might thus have a positive effect in CVD in RA, although it also has been described that IFNβ accelerates lesion formation in atherosclerotic mouse models.[65] In chapter 3.1 we studied the relation between *IRF5* genetics and carotid intima media thickness (cIMT), which is an early marker for atherosclerosis but does reflect plaque formation in only a minority of patients[66] and demonstrate that the genetic variant of *IRF5* rs2004640 G-allele is related to higher cIMT whereas the rs2004640-T allele is related to lower cIMT levels in RA in patients older than 60 years. Since the latter genetic variant of *IRF5* is known to relate to increased type I IFN levels it may therefor be involved in the atherosclerotic process in rheumatoid arthritis via enhanced production of type I IFNs. As have been described above, *IRF5* is a regulator of type I IFN-activity and functions as a transcription factor when phosphorylated, leading to expression of downstream interferon response genes as well as production of type I IFN itself. Furthermore, it is known that the *IRF5* isoforms differ in their ability to transactivate type I IFN genes, e.g. IFNα or IFNβ.[22] Thus the IRF5 rs2004640 T-allele is likely to enhance the expression of *IRF5* and successively type I IFN, including IFNβ, whereas the

G-allele is not. This is in line with the protective role of IFNβ in vascular diseases as described by Zhang et al. [43] They showed that IFNβ can play a prominent anti-atherosclerosis, anti-inflammation, and anti-proliferation role of vasculoprotection by reducing angiotensin II-accelerated increase in vascular smooth muscle cell proliferation and intimal hyperplasia.[43] Influence of IFNβ on IMT may thus especially be effective in situations with high angiotensin activity, because high angiotensin-converting enzyme activity is associated with IMT. It seems relevant to further explore the role of *IRF5* genetics and IFNβ production in RA patients in relation to a larger panel of cardiovascular disease-related parameters, including cIMT and plaque formation.

Altogether, these finding point to a role of the *IRF5* transcription pathway in atherosclerosis and might have implications for clinical practice and future therapies in RA patients suffering CV disease.

TYPE I IFN ACTIVITY IN IDIOPATIC INFLAMMATORY MYOPATIES

In patients with Idiopathic Inflammatory Myopaties, the activated type I interferon pathway has also been observed in a subset of patients, both at the tissue in e.g. muscle samples as well as in the periphery. Initial findings in IIM revealed that the presence of a type I IFN signature in the peripheral blood compartment correlated with disease activity.[44]

The relation between the presence of an type I IFN signature and a correlation to disease activity was also observed in SLE, but not other in autoimmune diseases like MS or RA.

In SLE, it has also been published that IFNα triggers this IFN signature and plays an important pathogenic role. In IIM however, evidence for a role of a particular type of IFN is lacking. An association with circulating IFNβ, but not IFNα or ω, levels was described for IIM[45] and according to others, IFNα levels appears to be reduced in IIM compared to healthy controls.[46] Despite these attempts to clarify the role of IFNs in IIM, the underlying mechanism remains unclear. Since SLE and IIM also share the presence of distinct autoantibodies and it is well known that some of the autoantibodies, those directed against nucleic-acid (RNA/DNA)-containing protein complexes, have a type I IFN inducing role in SLE, it is tempting to study whether similar associations between autoantibody specificities and the nature of the IFN signature as observed in SLE exist in IIM. In chapter 3.2 we investigated the relationship between the presence of autoantibodies directed against RNA binding proteins and other autoantibodies and the type I IFN signature in IIM. We found an association between the whole blood IFN signature in IIM and the presence of anti-RNA-binding proteins autoantibodies, such as Jo-1, U1RNP and Ro-60. Furthermore, an association between the presence of the IFN signature and multi-specific autoantibody profiles is observed. Moreover, we provide evidence that, in analogy with SLE, IFNα acts as an interferogenic trigger in the serum of IIM patients. These results point towards a strong relation between presence of autoantibodies against RNA-binding protein complexes and an IFNα driven type I IFN pathway activation

in IIM patients. Altogether, these findings suggest that the underlying mechanism for the activation of the type I IFN pathway in IIM is (partly) related to presence of RNA-binding autoantibodies in a similar way as has been described for SLE. These results might have implications for treatment and subclassification of these disorders.

THE PATHOGENIC ROLE OF TYPE I IFN ACTIVITY IN AUTOIMMUNE DISEASES

It is apparent that type I IFNs are in the center of attention in chronic inflammatory diseases. The type I IFN signature has now been observed in PB cells and lesional tissue of (a subset of) patients with different inflammatory diseases such as RA, SLE, scleroderma, Sjögren's syndrome, MS and type I diabetes. The heterogeneous gene expression profiles, especially with respect to type I IFN activity, of patients with clinically defined similar diseases are an exponent of the different intrinsic modes of immune status that may underlie these diseases. It also makes more evident the complexity of the diseases and the relation to therapy responsiveness, as described in this thesis.

An intriguing point is the opposite roles of type I IFNs in autoimmunity, e.g. in SLE and MS. Compelling evidence from studies in SLE demonstrated that in particular IFNα is directly implicated in the pathogenesis.[47,67] SLE is characterized by autoimmunity, exemplified by the presence of autoantibodies to nucleic acid and associated proteins, and organ inflammation. Serum levels of IFNα were increased in SLE and associated with disease severity and organ involvement. Supportive for the pathogenic role of IFNα in SLE was the observation that virally infected persons and cancer patients who were treated with IFNα sometimes produced anti-nuclear antibodies and occasionally developed SLE-like symptoms.[48] The mechanisms by which IFNα may promote autoimmunity may involve the induction of autoreactive lymphocytes, enhancement of long-term antibody responses, and priming of myeloid cells.

On the other hand type I IFNs are therapeutic in MS. Treatment with IFNß reduces clinical relapses, has an ameliorating effect on brain disease activity, and possibly slows down progression of disability. The anti-inflammatory and tissue protective mechanism of IFNβ likely involve antiproliferative and proapoptotic effects via a variety of molecular changes [49]Their anti-inflammatory effects also include the increased expression of immune dampening mediators such as IL-10, IL-1R antagonist and soluble TNF receptors, and inhibition of the production of pro-inflammatory mediators such as IL-1, IL-6 and TNFα.

In addition to the role of exogenous IFNβ, endogenous IFN was shown to play a role in the disease as well, since we observed heterogeneous activation of the type I IFN system. Endogenous activation of the type I IFN system is also related to the beneficial effect of

exogenous IFNβ. There is, however, no evidence of a relation between the type I IFN activity and disease activity in MS. It is in this perspective notable that genetic variation in *IRF5* is associated with an activated type I IFN system in both, SLE and MS patients, suggesting that the underlying mechanism might be the same.

In the other autoimmune diseases that show involvement of type I IFNs their role is less clear.

Our studies In IIM showed a correlation between the IFN signature and disease activity, similar to what was seen in SLE. Additionally, we revealed that autoantibody specificities are related to the presence of an IFN signature, as is true for SLE. We subsequently provided evidence for a similar role of autoantibody specificities in myositis and showed that, in line with SLE, IFNα is responsible for the observed IFN signature in IIM. This shed new light on subclassification of patients with IIM disorders and might as well have implications for treatment of patients. Currently, Phase II trials are ongoing for IFNα-targeting treatment (sifalimumab and rontalizumab) in SLE are ongoing and probably these therapies might be beneficial in the subset of IIM patients characterized by an activated type I IFN system as well.

In RA, the heterogeneous activation of the type I IFN signature has been described but no associations with disease parameters or activity could be shown. Up until now, there is therefor no evidence for a direct role of type I IFNs in the pathogenesis of this disease. Our studies however do clearly indicate that the a priori activation of the IFN system is related to the lack of beneficial effect of B-cell depletion and that the activation of type I IFN system through B-cell depletion is related to its beneficial effect. These associations to clinical response to rituximab are a major step towards a personalized medicine approach in RA, which is underlined by the high specificity and sensitivity and positive predictive value for treatment outcome. From a biological point of view, our data implicates that type I IFNs might contribute to the effect of B cell treatment in these patients and might thereby play a beneficial role in the disease. Additionally, our data suggest that *IRF5*-related activation of type I IFNs might be inhibitory for the development of cardiovascular disease in these patients. This relation between *IRF5* genetics and cardiovascular diseases in RA underscore the multifactorial role of type I IFN in autoimmunity.

From the above, the question emerges why type I IFNs are pathogenic in systemic autoimmune diseases like in SLE and beneficial in MS? Since this paradox is exemplified by a pathogenic role of IFNα in SLE and a beneficial role of IFNβ in MS it is tempting to speculate that IFNα and IFNβ have distinct roles in immunoregulation that confer these opposing effects.

Moreover, in RA potentially beneficial effects for IFNβ were claimed from studies using different animal models, which showed reduced disease severity in the presence of IFNβ,[50-55] while the lack of IFNβ (IFNβ knock-out) increased disease severity.[38,56]

Comparison between the primary amino acid sequences revealed that IFNα differs from IFNβ by approximately 70%.[57] Receptor binding studies demonstrated that IFNα and IFNβ interact with the receptor in a different manner, suggesting that IFNα and IFNβ activate the IFNAR1/IFNAR2 triggered signal transduction pathway in a slightly different way.[58-61] Accordingly, in-vitro studies revealed that IFNβ appeared to be more potent at inhibiting cell proliferation and apoptosis than IFNα.[62-64] However, until now differences in the downstream gene activation program, i.e. IFN signature, between IFNα and IFNβ are unknown.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our research showed the added value of genome wide transcription profiling in unraveling the biological mechanism underlying treatment response. New insights have emerged and our results place the type I IFN system in the center of attention in autoimmunity. Although many new questions arose about the exact role of this system in the different autoimmune diseases and its relation to treatment (non-)responsiveness in both, MS and RA, the prospect for their role as biomarkers are encouraging.

Our gene expression profiling studies in MS and RA pointed towards a dysregulated type I IFN pathway. In order to get insight in the possible dysregulation of these pathways we need to study the proteins of the type I IFN signaling pathway, both upstream and downstream IFN, as well as their phosphorylation status. Furthermore, it is relevant to search for the trigger that causes the elevated expression of type I IFN related genes, both, in untreated MS and RA patients as well as (RTX-)treated patients. Thereto, bioassays to determine the capacity of patients sera to induce type I IFN activity can be performed, as well as expression level analysis in cell subsets to determine which cells are responsible for the whole blood type I IFN signature.

The observed association between *IRF5* genetics and IMT in RA not only needs to be validated in larger cohorts but also the role of IFNβ in this association needs to be studied as well as the relation of *IRF5* genetics to plaque formation. If it is true that enhanced IMT in RA is not seen in patients with elevated levels of IFNβ, this might have consequences for the treatment of these patients in order to prevent cardiovascular disease. But not only the route via which *IRF5* genetics might influence IMT levels in RA is important to study. Also relation of *IRF5* genetics as well as type I IFN biology in general to a broader collection of cardiovascular related disease parameters needs to be determined in order to better understand type I IFN biology in RA-related cardiovascular disease.

The challenges we face now are dual. On the one hand, we are only starting to reveal the complex role of type I IFNs in autoimmunity. Since the molecular differences most likely reflect distinct pathophysiologic processes underlying disease, further research involving an integrated approach using genomics, genetics, molecular cell biology, signal transduction and proteomics, needs to be performed to better understand the physiological role of the type I IFNs with respect to disease and treatment response. On the other hand, our research resulted in reliable and easy to use biomarker to predict responsiveness to treatment. However, prospective studies are needed to further validate the clinical value of our biomarkers for IFNβ treatment in MS and rituximab treatment in RA. Subsequent rigorous technological validation and standardization are required before these classifiers can be implemented in clinical practice to predict individual responsiveness to treatment. Altogether, important steps have been taken towards personalized medicine for MS and RA patients.

REFERENCES

1 Comabella M, Lunemann JD, Rio J, Sanchez A, Lopez C, Julia E, Fernandez M, Nonell L, Camina-Tato M, Deisenhammer F, Caballero E, Tortola MT, Prinz M, Montalban X, Martin R. A type I interferon signature in monocytes is associated with poor response to interferon-β in multiple sclerosis. Brain 2009;132:3353-3365

2 Dupont SA, Goelz S, Goyal J, Green M. Mechanisms for regulation of cellular responsiveness to human IFN-beta1a. J Interferon Cytokine Res 2002; 22(4):491-501.

3 Bustamante MF, Fissolo N, Río J, Espejo C, Costa C, Mansilla MJ, Lizasoain I, Moro MA, Carmen Edo M, Montalban X, Comabella M. Implication of the Toll-like receptor 4 pathway in the response to interferon-β in multiple sclerosis. Ann Neurol. 2011;70(4):634-645.

4 Lehtonen A, Matikainen S, Julkunen I. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. J Immunol 1997; 159(2):794-803.

5 Gresser I. Biologic effects of interferons. J Invest Dermatol 1990; 95(6 Suppl):66S-71S.

6 Palucka AK, Blanck JP, Bennett L, Pascual V, Banchereau J. Cross-regulation of TNF and IFNα in autoimmune diseases. Proc Natl Acad Sci U S A. 2005;102(9):3372-7.

7 Traugott U, Lebon P. Multiple sclerosis: involvement of interferons in lesion pathogenesis. Ann Neurol 1988;24:243-251;

8 Lande R, Gafa V, Serafini B, Glacomini E, Visconti A, Remoli ME, Severa M, Parmentier M, Ristori G, Salvetti M, Aloisi F, Coccia EM. Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-β. J Neuropathol Exp Neurol 2008;67:388-401

9 Jenner RG, Young RA. Insights into host responses against pathogens from transcriptional profiling. Nat Rev Microbiol 2005;3:281-294

10 van Baarsen LG, van der Pouw Kraan TC, Kragt JJ, Baggen JM, Rustenburg F, Hooper T, Meilof JF, Fero MJ, Dijkstra CD, Polman CH, Verweij CL. A subtype of multiple sclerosis defined by an activated immune defense program. Genes Immun. 2006 Sep;7(6):522-31.

11 Lovgren T, Eloranta ML, Bave U, Alm GV, Ronnblom L. Induction of interferon-α production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. Arthrtis Rheum 2004;50:1861-1872

12 Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, Naves R, Han M, Zhong F, Castellanos JG, Mair R, Christakos A, Kolkowitz I, Katz L, Killestein J, Polman CH, de Waal Malefyt R, Steinman L, Raman C. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. Nat Med. 2010;16(4):406-12.

13 Bushnell SE, Zhao Z, Stebbins CC, Cadavid D, Buko AM, Whalley ET, Davis JA, Versage EM, Richert JR, Axtell RC, Steinman L, Medori R. Serum IL-17F does not predict poor response to IM IFNβ IFNβ-1a in relapsing-remitting MS. Neurology. 2012 May 9. [Epub ahead of print]

14 Barnes BJ, Kellum MJ, Pinder KE, Frisancho JA, Pitha PM. Interferon regulatory factor 5, a novel mediator of cell cycle arrest and death. Cancer Res 2003;63:6424-6431

15 Schoenemeyer A, Barnes BJ, Mancl ME, Latz ME, Goutagny N, Pitha PM et al. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor signalling. J Biol Chem 2009;284:2767-2777

16 Hu G, Barnes BJ. IRF5 is a mediator of the death receptor-induced apoptotic signalling pathway. J Biol Chem 2009;284:2767-2777

17 Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythemathosus. Nat Genet 2006;38:550-555

18 Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. Curr Opin Immunol 2006;18:676-682

19 Rullo OJ, Woo JM, Wu H, Hoftman AD, Maranian P, Brahn BA et al. Association of IRF5 polymorphisms with activation of the interferon-α pathway. Ann Rheum Dis 2010;69:611-617

20 Honda K, Yanai H, Takaoka A, Taniguchi T. Regulation of the type I IFN induction: a current view. Int Immunol. 2005 Nov;17(11):1367-78. Epub 2005 Oct 7. Review. PubMed PMID: 16214811.

21 Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, Ortmann WA, Koeuth T, González Escribano MF; Argentine and Spanish Collaborative Groups, Pons-Estel B, Petri M, Daly M, Gregersen PK, Martín J, Altshuler D, Behrens TW, Alarcón-Riquelme ME. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet. 2006 May;38(5):550-5.

22 Mancl ME, Hu G, Sangster-Guity N, Olshalsky SL, Hoops K, Fitzgerald-Bocarsly P, Pitha PM, Pinder K, Barnes BJ. Two discrete promoters regulate the alternatively spliced human interferon regulatory factor-5 isoforms. Multiple isoforms with distinct cell type-specific expression, localization, regulation, and function. J Biol Chem. 2005 Jun 3;280(22):21078-90

23 Quartuccio L, Fabris M, Salvin S, Atzeni F, Saracco M, Benucci M, Cimmino M, Morassi P, Masolini P, Pellerito R, Cutolo M, Puttini PS, De Vita S: Rheumatoid factor positivity rather than anti-CCP positivity, a lower disability and a lower number of anti-TNF agents failed are associated with response to rituximab in rheumatoid arthritis

24 Dass S, Rawstron AC, Vital EM, Henshaw K, McGonagle D, Emery P. Highly sensitive B cell analysis predicts response to rituximab therapy in rheumatoid arthritis. Arthritis Rheum 58:2993-9, 2008

25 Roll P, Palanichamy A, Kneitz C, Dorner T, HP Tony. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. Arthritis Rheum 54:2377–86, 2006

26 Nakou M, Katsikas G, Sidiropoulos P, Bertsias G, Papadimitraki E, Raptopoulou A, Koutala H, Papadaki HA, Kritikos H, Boumpas DT. Rituximab therapy reduces activated B cells in both the peripheral blood and bone marrow of patients with rheumatoid arthritis: depletion of memory B cells correlates with clinical response. Arthritis Res Ther 11: R131, 2009.

27 Möller B, Aeberli D, Eggli S, Fuhrer M, Vajtai I, Vögelin E, Ziswiler HR, Dahinden CA, Villiger PM. Class-switched B cells display response to therapeutic B-cell depletion in rheumatoid arthritis. Arthritis Research & Therapy 11:R62, 2009

28 Fabris M, Quartuccio L, Saracco M. BLyS promoter polymorphism and response to rituximab in rheumatoid arthritis patients positive or negative for rheumatoid factor. Arthritis Rheum 60:S626-S627 (abstract), 2009

29 Julià A, Barceló M, Erra A, Palacio C, Marsal S. Identification of candidate genes for rituximab response in rheumatoid arthritis patients by microarray expression profiling in blood cells. Pharmacogenomics 10:1697-708, 2009

30 Magnusson M, Brisslert M, Zendjanchi K, Lindh M, Bokarewa MI. Epstein-Barr virus in bone marrow of rheumatoid arthritis patients predicts response to rituximab treatment. Rheumatology 49:1911-9, 2010

31 Quartuccio L, Lombardi S, Fabris M, Masolini P, Saracco M, Pellerito R, De Vita S. Long-term effects of rituximab in rheumatoid arthritis: clinical, biologic and pharmacogenetic aspects. Ann N Y Acad Sci 1173:692-700, 2009

32 De la Torre, Moura RA, Leandro MJ, Edwards J, Cambridge G. B-cell-activating factor receptor expression on naive and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B-cell depletion therapy. Ann Rheum Dis 69:2181-8, 2010.

33 Fabre, Guisset C, Tatem L, Dossat N, Dupuy AM, Cohen JD, Cristol AM, Daures JP, Jorgensen C. Protein biochip array technology to monitor rituximab in rheumatoid arthritis. Clin Exp Immunol 155:395-402, 2008

34 Thurlings RM, Vos K, Wijbrandts CA, Zwinderman AH, Gerlag DM, Tak PP. Synovial tissue response to rituximab: mechanism of action and identification of biomarkers of response. Ann Rheum Dis 67:917-25, 2008

35 Interpreting Diagnostic Tests. http://gim.unmc.edu/dxtests/Default.htm

36 Cantaert T, van Baarsen LG, Wijbrandts CA, Thurlings RM, van de Sande MG, Bos C, van der Pouw TK, Verweij CL, Tak PP, Baeten DL. Type I interferons have no major influence on humoral autoimmunity in rheumatoid arthritis. Rheumatology 49:156–66, 2010

37 Jego G, Pascual V, Palucka AK, Banchereau J. Dendritic cells control B cell growth and differentiation. Curr Dir Autoimmun 8:124-39, 2005

38 Treschow AP, Teige I, Nandakumar KS, Holmdahl R, Issazadeh-Navikas S. Stromal cells and osteoclasts are responsible for exacerbated collagen-induced arthritis in interferon-beta-deficient mice. Arthritis Rheum 52:3739-48, 2005

39 De Hooge AS, van de Loo FA, Koenders MI, Bennink MB, Arntz OJ, Kolbe T, van den Berg WB. Local activation of STAT-1 and STAT-3 in the inflamed synovium during zymosan-induced arthritis: exacerbation of joint inflammation in STAT-1 gene-knockout mice. Arthritis Rheum 50:2014-23, 2004

40 Van Holten HJ, Plater-Zyberk C, Tak PP. Interferon-beta for treatment of rheumatoid arthritis? *Arthritis Res Therapy* 4:346-52, 2002

41 Merrill JT, Neuwelt CM, Wallace DJ et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum* 62:222-33, 2010

42 Grisar J, Aletaha D, Steiner CW, Kapral T, Steiner S, Seidinger D, Weigel G, Schwarzinger I, Wolozcszuk W, Steiner G, Smolen JS. Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis. Circulation. 2005 Jan 18;111(2):204-11

43 Zhang LN, Velichko S, Vincelette J, Fitch RM, Vergona R, Sullivan ME, Croze E, Wang YX. Interferon-beta attenuates angiotensin II-accelerated atherosclerosis and vascular remodeling in apolipoprotein E deficient mice. Atherosclerosis. 2008 Mar;197(1):204-11

44 Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R, Barohn RJ, Saperstein DS, Briemberg HR, Ericsson M, Park P, Amato AA. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. Ann Neurol. 2005 May;57(5):664-78

45 Liao AP, Salajegheh M, Nazareno R, Kagan JC, Jubin RG, Greenberg SA Interferon β is associated with type 1 interferon-inducible gene expression in dermatomyositis. Ann Rheum Dis. 2011 May;70(5):831-6. doi: 10.1136/ard.2010.139949. Epub 2010 Dec 21.

46 Król P, Kryštůfková O, Polanská M, Mann H, Klein M, Beran O, Vencovský J Serum levels of interferon α do not correlate with disease activity in patients with dermatomyositis/polymyositis. Ann Rheum Dis. 2011 May;70(5):879-80. doi: 10.1136/ard.2010.141051. Epub 2010 Nov 10.

47 Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science. 2001 Nov 16;294(5546):1540-3.

48 Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity. 2006 Sep;25(3):383-92. Review.

49 Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. Apoptosis 2003; 8(3):237-249.)

50 Adriaansen J, Kuhlman RR, van Holten J, Kaynor C, Vervoordeldonk MJ, Tak PP. Intraarticular interferon-beta gene therapy ameliorates adjuvant arthritis in rats. Hum Gene Ther. 2006 Oct;17(10):985-96.

51 Katakura K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest. 2005 Mar;115(3):695-702. Erratum in: J Clin Invest. 2005 Apr;115(4):1100.

52 Corr M, Boyle DL, Ronacher L, Flores N, Firestein GS. Synergistic benefit in inflammatory arthritis by targeting I kappaB kinase epsilon and interferon beta. Ann Rheum Dis. 2009 Feb;68(2):257-63.

53 Yarilina A, DiCarlo E, Ivashkiv LB. Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs. J Immunol. 2007 Feb 15;178(4):2204-11.

54 van Holten J, Reedquist K, Sattonet-Roche P, Smeets TJ, Plater-Zyberk C, Vervoordeldonk MJ, Tak PP. Treatment with recombinant interferon-beta reduces inflammation and slows cartilage destruction in the collagen-induced arthritis model of rheumatoid arthritis. Arthritis Res Ther. 2004;6(3):R239-49.

55 Triantaphyllopoulos KA, Williams RO, Tailor H, Chernajovsky Y. Amelioration of collagen-induced arthritis and suppression of interferon-gamma, interleukin-12, and tumor necrosis factor alpha production by interferon-beta gene therapy. Arthritis Rheum. 1999 Jan;42(1):90-9.

56 Guo B, Chang EY, Cheng G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. J Clin Invest. 2008 May;118(5):1680-90.

57 Taniguchi T, Mantei N, Schwarzstein M, Nagata S, Muramatsu M, Weissmann C. Human leukocyte and fibroblast interferons are structurally related. Nature. 1980 Jun 19;285(5766):547-9.

58 Runkel L, Pfeffer L, Lewerenz M, Monneron D, Yang CH, Murti A, Pellegrini S, Goelz S, Uzé G, Mogensen K. Differences in activity between alpha and beta type I interferons explored by mutational analysis. J Biol Chem. 1998 Apr 3;273(14):8003-8

59 Platis D, Foster GR. Activity of hybrid type I interferons in cells lacking Tyk2: a common region of IFN-alpha 8 induces a response, but IFN-alpha2/8 hybrids can behave like IFN-beta. J Interferon Cytokine Res. 2003 Nov;23(11):655-66

60 Abramovich C, Shulman LM, Ratovitski E, Harroch S, Tovey M, Eid P, Revel M. Differential tyrosine phosphorylation of the IFNAR chain of the type I interferon receptor and of an associated surface protein in response to IFN-alpha and IFN-beta. EMBO J. 1994 Dec 15;13(24):5871-7

61 Lewerenz M, Mogensen KE, Uzé G. Shared receptor components but distinct complexes for alpha and beta interferons. J Mol Biol. 1998 Sep 25;282(3):585-99

62 Chawla-Sarkar M, Leaman DW, Borden EC. Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. Clin Cancer Res. 2001 Jun;7(6):1821-31.

63 Leaman DW, Chawla-Sarkar M, Jacobs B, Vyas K, Sun Y, Ozdemir A, Yi T, Williams BR, Borden EC. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. J Interferon Cytokine Res. 2003 Dec;23(12):745-56

64 Johns TG, Mackay IR, Callister KA, Hertzog PJ, Devenish RJ, Linnane AW. Antiproliferative potencies of interferons on melanoma cell lines and xenografts: higher efficacy of interferon beta. J Natl Cancer Inst. 1992 Aug5;84(15):1185-90

65 Goossens P, Gijbels MJ, Zernecke A, Eijgelaar W, Vergouwe MN, van der Made I, Vanderlocht J, Beckers L, Buurman WA, Daemen MJ, Kalinke U, Weber C, Lutgens E, de Winther MP. Myeloid type I interferon signaling promotes atherosclerosis by stimulating macrophage recruitment to lesions. Cell Metab. 2010 Aug 4;12(2):142-53

66 Arbeille P, Desombre C, Aesh B, Philippot M, Lapierre F. Quantification and assessment of carotid artery lesions: degree of stenosis and plaque volume. J Clin Ultrasound. 1995 Feb;23(2):113-24

67 Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum. 2005 May;52(5):1491-503.