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INHIBITION OF INNATE IMMUNE  
ACTIVATION BY  
MODIFICATIONS IN BACTERIAL RNA

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**LIST OF ABBREVIATIONS**

|               |  |
|---------------|--|
| AIM2          | absent in melanoma 2   |
| AlphaScreen™  | amplified luminescent proximity homogenous binding assay screen      |
| ASC           | apoptosis-associated speck-like protein containing a CARD            |
| ATP           | adenosine triphosphate   |
| bp            | base pair(s)   |
| bRNA          | bacterial RNA  |
| CARD          | Caspase activation and recruitment domain                            |
| cGAS          | cyclic GAMP synthetase   |
| CHO           | Chinese hamster ovary cells  |
| CLR           | C-type binding domains-like receptor                                 |
| DAMP          | danger or damage associated molecular pattern                        |
| DC            | dendritic cell   |
| DEPC          | diethylpyrocarbonat  |
| DExD/H        | aspartate-glutamate-any amino acid-aspartate/histidine box helicases |
| DMEM          | Dulbecco's Modified Eagle Medium                                     |
| DNA           | deoxyribonucleic acid  |
| ds            | double strand  |
| <i>E.coli</i> | <i>Escherichia coli</i>  |
| ELISA         | Enzyme-linked Immunosorbent Assay                                    |
| ER            | endoplasmatic reticulum  |

|                       |  |
|-----------------------|--|
| <i>erm</i>            | erythromycin resistance methylases               |
| FCS                   | fetal calf serum                                 |
| Fig.                  | Figure   |
| GM-CSF                | granulocyte-macrophage colony stimulating factor |
| h                     | hour(s)  |
| HMGB                  | high mobility group box                          |
| HRP                   | horseradish peroxidase                           |
| HSP                   | heat shock protein                               |
| IBD                   | inflammatory bowel disease                       |
| IFI                   | Interferon inducible protein                     |
| IFN                   | interferon                                       |
| I $\kappa$ B $\alpha$ | inhibitor of NF $\kappa$ B- $\alpha$             |
| IL                    | interleukine                                     |
| IRAK                  | Interleukin-1 receptor-associated kinase         |
| IRF                   | interferon regulatory factor                     |
| ISG                   | interferon-stimulated genes                      |
| LGP2                  | laboratory of genetics and physiology 2          |
| LPS                   | lipopolysaccharide                               |
| LRR                   | leucine-rich repeats                             |
| MAL                   | MyD88-adaptor-like protein                       |

|                         |  |
|-------------------------|--|
| MAPK                    | Mitogen-activated protein kinase                 |
| MAVS                    | mitochondrial antiviral-signal protein           |
| MCMV                    | murine cytomegalovirus                           |
| MDA5                    | melanoma differentiation-associated gene 5       |
| min                     | minute(s)  |
| miRNA                   | micro RNA  |
| mRNA                    | messenger RNA                                    |
| MyD88                   | Myeloid differentiation primary response gene 88 |
| NaCl                    | sodiumchloride                                   |
| NaF                     | sodiumfluoride                                   |
| NF $\kappa$ B- $\alpha$ | nuclear factor $\kappa$ B- $\alpha$              |
| NOD                     | nucleotide-binding oligomerization domain        |
| NLR                     | NOD-like receptor                                |
| PAMP                    | pathogen associated molecular pattern            |
| PBMCs                   | peripheral blood mononuclear cells               |
| pDC                     | plasmacytoid dendritic cell                      |
| PRR                     | pattern recognition receptor                     |
| R-genes                 | plant resistance genes                           |
| rE                      | relative expression                              |
| RIG-I                   | retinoic acid-inducible gene 1                   |
| RLR                     | RIG-I-like receptor                              |
| RNA                     | ribonucleic acid                                 |
| rRNA                    | ribosomal RNA                                    |

|                     |  |
|---------------------|--|
| RT                  | room temperature   |
| SARM                | sterile- $\alpha$ and Armadillo motif-containing protein   |
| SDS                 | sodium dodecyl sulphate                                    |
| SDS-PAGE            | SDS-polyacrylamid gel electrophoresis                      |
| SLE                 | systemic lupus erythematosus                               |
| ss                  | single strand  |
| STING               | Stimulator of interferon genes                             |
| TIR                 | toll/interleukin-1 receptor                                |
| TLR                 | toll-like receptor   |
| TNF                 | tumor necrosis factor                                      |
| TRAM                | translocating chain-associating membrane protein           |
| TRIF                | TIR-domain-containing adapter-inducing interferon- $\beta$ |
| trmA                | uracil-5-methyltransferase                                 |
| trmB                | guanosine-7-methyltransferase                              |
| trmH                | Gm18-2'-O-methyltransferase                                |
| tRNA                | transfer RNA   |
| tRNA <sup>Tyr</sup> | tRNA for tyrosine  |
| $\Psi$              | pseudouridine  |

# 1 SUMMARY

Modification of RNA is an important function in the immunological discrimination between self and non-self RNA. Naturally occurring RNA modifications have been identified that suppress activation of the innate immune response, e.g. 2'O-methylation of the ribose backbone. It has already been shown that incorporation of 2'O-methylation in siRNA abrogates TLR7-dependent interferon-alpha production in human plasmacytoid dendritic cells (pDCs). In the present study, a native bacterial tRNA with 2'O-methylation at position G18 was analysed. It was found that the 2'O-methylation not only made the tRNA non-stimulatory, but also led to dominant inhibitory effects. Thus, this modification impaired immunostimulation by RNA species that would otherwise have been stimulatory. Further analysis revealed that not only was interferon alpha production by pDCs via TLR7 suppressed, but also secretion of pro-inflammatory cytokines by monocytes, including IL12p40 and TNF. Monocytes express a different subset of nucleic acid-sensing receptors, indicating that 2'O-methylated RNA influences the stimulation of different receptors. Analysis of other TLR ligands showed that the inhibitory effect was specific for stimulation by RNA but it was not observed for R848, a synthetic small molecule agonist of TLR7/8, or other TLR ligands. Investigation of the signalling pathway in response to RNA stimulation showed that modified, inhibitory RNA suppresses TLR stimulation at proximal levels. Preliminary binding studies indicated that 2'O-methylated RNA can bind directly to TLR7; it does not induce signalling but displaces unmethylated stimulatory RNA. As 2'O-methylation of tRNA at position G18 is found in tRNAs from many species and the neighbouring sequences are conserved, the influence of location and the whole sequence motif itself were investigated. The location of the 2'O-modification turned out to be of minor importance, but the inhibitory capacity of modified tRNA was influenced by the sequence of the motif. The sequence motif DmR was found to be necessary for immunosilencing of the tRNA, where D is a base other than cytosine with a 2'O-methyl position in the ribose of the nucleoside and R is a purine (adenine or guanine) immediately upstream of the methylation (position +1).

Together, the results show that 2'O-methylation of RNA in a defined sequence motif abrogates immunostimulation in different cell types. Naturally occurring RNA modifications serve to distinguish self RNA from foreign RNA. The results from this thesis can be used to reduce immunostimulation by any RNA when immune stimulation is not intended.

## 2 ZUSAMMENFASSUNG

Natürlich vorkommende Modifikationen spielen eine wichtige Rolle bei der Unterscheidung zwischen eigener und fremder RNA. Verschiedene Modifikationen, die in der Lage sind, eine Immunantwort zu unterdrücken, wurden identifiziert. Eine von ihnen ist die 2'-O-Methylierung der Ribose, welche, eingebaut in siRNAs, in der Lage ist die Interferon-alpha Produktion von plasmacytoiden Dendritischen Zellen (pDCs) durch TLR7-Aktivierung zu unterdrücken. In der vorliegenden Arbeit wurde eine bakterielle tRNA untersucht, die an der Position G18 eine natürlich vorkommende 2'-O-Methylierung aufweist. Diese Modifikation führte nicht nur dazu, dass die tRNA alleine keine Interferon-alpha Produktion mehr aktiviert, sondern diese modifizierte tRNA und Bruchstücke daraus sind auch in der Lage, die Aktivierung durch stimulative RNA zu inhibieren. Es wurde weiter gezeigt, dass diese Inhibition nicht nur die Produktion von Interferon-alpha aus pDCs über TLR7 unterdrückt, sondern auch anderer pro-inflammatorischer Zytokine wie IL12p40 und TNF aus Monozyten, welche andere Nukleinsäure-erkennende Rezeptoren als pDCs exprimieren. Dies weist darauf hin, dass die Stimulation verschiedener Rezeptoren durch modifizierte RNA unterdrückt werden kann. Der Effekt ist allerdings spezifisch für die Stimulation durch RNA, da die Stimulation durch R848, einen synthetischen TLR7/8 Agonisten, oder durch andere TLR Liganden nicht inhibiert werden konnte. Untersuchungen auf der Ebene der Signalweiterleitung zeigten eine Inhibition durch modifizierte RNA bereits proximal in der Signalkaskade. Daher wurde die direkte Bindung von 2'-O-methylierter RNA an TLR7 untersucht. Erste Studien zeigten, dass 2'-O-methylierte RNA direkt an den Rezeptor bindet und nicht modifizierte, stimulative RNA verdrängt. Da 2'-O-Methylierung an G18 in verschiedenen tRNAs beobachtet wurde, wurde der Einfluss von Position und Sequenzmotiv auf den inhibitorischen Effekt untersucht. Dabei konnte gezeigt werden, dass weniger die Lokalisation der Modifikation innerhalb der tRNA sondern vielmehr die Sequenz um die Modifikation den Effekt beeinflusst. Das Sequenzmotive DmR wurde als das ausschlaggebende für die Inhibition identifiziert. Dabei ist D eine beliebige Base außer Cytosin mit der 2'-O-Methylierung und R ist ein Purin an der Position +1 in 3' Richtung von der 2'-O-methylierten Position. Zusammenfassend hat diese Studie gezeigt, dass RNA mit einer 2'-O-Methylierung in einem definierten Sequenzkontext, die Immunstimulation in verschiedenen Zellarten unterdrückt. Natürlich vorkommende 2'-O-Methylierungen werden zur Selbst/Fremd Diskriminierung verwendet. Mit den Ergebnissen dieser Arbeit können durch Einführung von 2'-O-Methylierungen beliebige RNA Spezies inhibierend gemacht werden.



### 3 INTRODUCTION

The immune system is important for the recognition and clearance of pathogens. To function in a proper way, the immune system has to cope with a wide range of pathogens - from viruses and bacteria to fungi and parasites. Thus, an important characteristic is to be able to distinguish between self and foreign structures. The immune system has two main parts, the innate immune system and the adaptive immune system. In evolutionary terms, the innate immune system is the older of the two. It is found in all animals and plants, and is the organism's first line of defence. The adaptive immune system is activated through antigen-presenting cells of the innate immune system and is based on clonal selection of lymphocytes. The response is specific and results in formation of immune memory.

#### 3.1 Recognition within the innate immune system

Invading pathogens must be quickly recognised by the immune system. This important function is fulfilled by germline-encoded receptors, which have been identified as pattern recognition receptors (PRRs). PRRs detect highly conserved components of microbes, so-called pathogen-associated molecular patterns (PAMPs)<sup>2</sup>. PAMPs are essential to microbes, and they cannot be easily manipulated<sup>2,3</sup>. Thus, a broad range of pathogens can be recognised with a limited number of different receptors<sup>2,4,5</sup>.

PRRs fall into different groups according to their molecular structure: Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), interferon-inducible protein (Ifi), absent in melanoma 2 (Aim2)-like receptors, and receptors with C-type binding domains (CLRs)<sup>6-11</sup>. These receptors are located at the cell surface, in intracellular compartments, or in the cytosol. All of them are composed of a ligand binding domain and a signalling domain. Upon binding of the ligand, the signalling domain is activated mostly by dimerisation, oligomerization, or conformational changes. Then the signalling domain recruits adapter proteins to initiate intracellular signalling.

Cytosolic receptors are expressed in most cell types, while TLRs are mostly expressed in immune cells. Innate immune cells include phagocytic cells, antigen-presenting cells, natural killer cells, mast cells, and platelets. Phagocytic cells are mainly involved in the defence against extracellular microbes. The group of phagocytic cells includes monocytes, macrophages, and polymorphonuclear granulocytes. Monocytes circulate in the blood but can be recruited into

various tissues upon infection, where they differentiate into macrophages. Macrophages can also be found as steady-state cells in most tissues. Antigen-presenting cells are another group of immune cells. They fall into different groups, depending on their origin and function. One group is made up of myeloid dendritic cells (DCs). They have a myeloid progenitor and express interleukin 8 (IL8) and IL12 as characteristic cytokines. On the other hand, plasmacytoid DCs (pDCs) have a lymphoid progenitor and mainly produce type-I interferons (IFNs).

### 3.1.1 Toll-like receptors

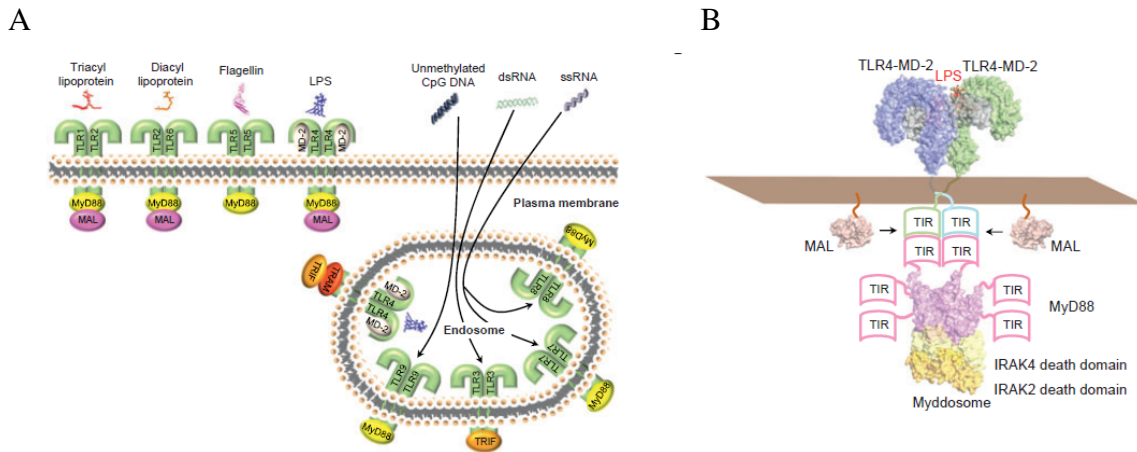
TLRs can activate the immune response to bacterial, fungal, and viral infections. They are homologous to the protein Toll in *Drosophila melanogaster*. Toll was first described by Christiane Nüsslein-Volhard in 1985 concerning its role in embryonic development<sup>12</sup>. In 1996, Jules A. Hoffmann and colleagues ascribed to Toll a role in immunity<sup>13</sup> when they observed that a fruit fly mutated in *toll* was no longer able to eliminate fungal infections. In 1998, Bruce Beutler and colleagues discovered the first ligand for a mammalian TLR, i.e. lipopolysaccharide (LPS) as ligand for TLR4<sup>14</sup>. Both Hoffmann and Beutler were honoured with the Nobel Prize in Medicine and Physiology in 2011 for “their discoveries concerning the activation of innate immunity” (The Nobel Assembly at Karolinska Institutet)<sup>15</sup>.

Currently, ten TLRs are known in humans (TLR1–TLR10), whereas in the murine system 12 receptors (TLR1–TLR9 and TLR11–TLR13) are known<sup>16</sup>. These are either expressed on the cell surface (TLR1, 2, 4, 5, 6, and 10) or in intracellular compartments (TLR3, 7, 8, and 9), namely endosomes<sup>17–20</sup>. Each TLR binds to a distinct family of microbial molecules and activates immune responses<sup>21,22</sup>. Activated TLRs form homo- or heterodimers. TLR2 builds heterodimers with TLR1 or TLR6 and recognises bacterial lipoproteins<sup>23–26</sup>. TLR4 complexes with MD2 to sense LPS<sup>14,27</sup>, and homodimers of TLR5 recognise the depolymerized form of flagellin<sup>28</sup>. The receptors TLR3, TLR7–TLR9, and TLR13 (the latter only in the murine system) sense various forms of bacterial and viral nucleic acids<sup>29–37</sup>. All the human TLRs with their ligands and adapter molecules are shown in Figure 3-1A.

Each TLR consists of an extracellular or luminal domain and an intracellular/cytosolic domain, which are connected by a transmembrane domain. The extracellular/luminal domain of TLRs forms a characteristic horseshoe-like structure of leucine-rich repeats (LRRs) - including some irregular loops which are thought to stick out from the LRR backbone, and which are important for ligand binding<sup>38–41</sup>.

The cytosolic domains of all TLRs share a TIR (Toll/interleukin-1 receptor) domain. This domain is also present in several signalling adapters. Activation of the receptor by ligand binding induces aggregation of the receptor with its adapter molecules mediated by homo- and heterotypic interaction between the TIR domains of receptor and adapter molecule<sup>42-47</sup>. In the human system, five TIR-domain-containing adapter molecules are involved: MyD88 (myeloid differentiation primary response gene 88), MAL (MyD88-adapter-like protein), TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ ), TRAM (translocating chain-associating membrane protein), and SARM (sterile- $\alpha$  and armadillo motif-containing protein)<sup>48-52</sup>. MyD88 is required for all TLRs except TLR3, which uses TRIF. TLR4 can signal via both MyD88 and TRIF. MAL is a bridging adapter essential for MyD88-dependent signalling of TLR2 and TLR4. TRAM is a bridging adapter for TRIF, and is necessary for TLR4 signalling but not for TLR3 signalling. SARM is probably a negative regulator of TLR signalling. It interacts with TRIF and therefore blocks its function. In addition to the TIR domain, MAL contains an N-terminal phosphatidyl inositol-binding motif and TRAM contains an additional myristoylation site. Both motifs are essential for membrane localisation of the molecules<sup>53,54</sup>. MyD88 contains a death domain required for the activation of downstream effectors. Interleukin-1 receptor-associated kinase (IRAK)-4 and IRAK1/IRAK2 are downstream kinases recruited directly to the TLR-MyD88 complex. The resulting large helical complex has been given the name 'Myddosome' (Figure 3-1B)<sup>46</sup>. The Myddosome has an indispensable role in TLR signalling. Its formation brings the kinase domains of the IRAKs into proximity and allows their phosphorylation and activation. In the next step, three different pathways are activated: phosphorylation of mitogen-activated protein kinase (MAPKs), activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) by phosphorylation of I $\kappa$ B- $\alpha$  (inhibitor of NF $\kappa$ B), and activation of IRF transcription factors. All three pathways can lead to the production of pro-inflammatory cytokines and interferons, which are important mediators of inflammation, immune regulation, survival, and proliferation.

The full-length TLR proteins containing the transmembrane domain are difficult to crystallise. Thus, to date, all structural studies have been done with truncated extracellular or intracellular domains; no full-length information is yet available. There are indications that activated and dimerised TLRs form clusters of higher order in the cell membrane. However, these structures have never been characterised in detail, since crystallisation of the whole complex has not yet been successful.



**Fig. 3-1: Toll-like receptor signalling complexes.** (A) Different TLRs with recruited signalling adaptors and ligands. (B) Speculated structure of TLR4-MD2-LPS-Myddosome complex <sup>1</sup>.

### 3.1.2 Cytosolic receptors

While TLRs are expressed in immune cells such as macrophages and DCs, cytosolic receptors such as RLRs and NLRs are also expressed in a broad range of somatic cells.

The RLR family has three members: RIG-I, MDA5 (melanoma differentiation-associated gene 5), and LGP2 (laboratory of genetics and physiology 2). RLRs were originally described as being RNA helicases. RIG-I and MDA5 recognise viral RNA and initiate an immune response. The activation leads to the production of pro-inflammatory cytokines and type-I IFN. A tandem domain of CARD (caspase activation and recruitment domain) is located at the N-termini of RIG-I and MDA5; it acts as a protein interaction motif by associating with downstream signalling components <sup>55</sup>. The CARD domain is missing in LGP2, so it is still being debated whether it functions as a positive or a negative regulator of RLR signalling <sup>55,56</sup>.

The central part of all three members of the RLR family shows some homology to the DExD/H box RNA helicase family members, and is responsible for the binding of double-stranded RNA and for ATP-dependent unwinding <sup>57</sup>. The signal following activation of RLRs is mediated by mitochondrial antiviral-signalling protein (MAVS, also known as IPS-1, VISA, and CARDIF), an adapter molecule with a function analogous to that of MyD88 for TLR signalling <sup>58-61</sup>. MAVS is attached to the mitochondrial membrane by the C-terminus and induces a delayed response of interferon-stimulated genes (ISGs) via IRF3 <sup>60</sup>. It has also been reported to be attached to peroxisomes <sup>62</sup>, where it enables the early induction of ISGs via interferon regulatory factor 1 (IRF1).

The NLRs are the largest group of cytosolic receptors. They have a tripartite structure, sharing high functional and structural similarities with plant resistance genes (R-genes) <sup>6,63-65</sup>. All NLRs contain a NACHT domain, which is responsible for oligomerization and activation. The phylogenetic history of the NACHT domains has been used for classification of NLRs <sup>6</sup>. The NLR family falls into two groups: NODs and NLRPs <sup>6,63,66</sup>. NODs mediate their signalling by an N-terminal CARD and they activate NFκB in a way that is similar to TLR and RLR signalling <sup>8,64,67</sup>. NLRPs, on the other hand, have an N-terminal pyrin effector domain and are able to activate caspase-1 <sup>6</sup>. NLRPs reside in the cytosol in an inactive and repressed form. Upon activation, the proteins form multimeric protein complexes, so-called inflammasomes, which are composed of the NLRP, an adapter molecule like Asc, and pro-caspase-1 <sup>68-70</sup>. Inflammasome formation leads to the proteolytic self-cleavage of pro-caspase-1 into the active form, which in turn cleaves pro-IL1β and pro-IL18 into the biologically active cytokines <sup>71</sup>.

Apart from NLRPs, inflammasome activation can also be induced by Absent in melanoma 2 (AIM2), which recognises foreign cytoplasmatic double-stranded DNA <sup>72,73</sup>.

STING has been shown to be an important adapter molecule for different cytosolic DNA receptors and to be critical for type-I interferon production upon detection of cytosolic DNA <sup>74-77</sup>. STING is also capable of directly sensing cyclic dinucleotides, which is used by bacteria as a second messenger <sup>78-82</sup>. Recently, cyclic 2'-5'-linked dinucleotides were found to be sensed by STING. They are synthesised by cGAMP synthetase (cGAS) after interaction of the enzyme with DNA <sup>83-87</sup>.

Other cytosolic receptors that sense DNA are IFI16 and members of the DExD/H box helicase family. IFI16 binds DNA in a non-sequence-specific manner through the negatively charged sugar-phosphate backbone of the DNA. It is coupled to STING signalling and leads to type-I interferon production <sup>88</sup>. The family of aspartate-glutamate-any amino acid-aspartate/histidine box helicases (DExD/H) contains important players in sensing of DNA. DHX36 and DHX9 sense CpG-DNA and lead to the production of type-I interferon and of pro-inflammatory cytokines, respectively <sup>89,90</sup>.

## 3.2 Pathogen-associated molecular patterns

To identify invading pathogens, the innate immune system senses PAMPs. These molecules are highly conserved and expressed by broad classes of pathogens, but they are not expressed by the host. Most PAMPs are expressed on the surfaces of microbes - such as lipoteichoic acid in gram-positive bacteria and LPS in gram-negative bacteria, which are recognised by TLR2 and TLR4 respectively<sup>14,91,92</sup>. Nucleic acids are exceptions to this rule. They are common to both the host and the pathogen and yet they have important roles in sensing of viruses and other microbes. Thus, special mechanisms have evolved to be able to distinguish "self" nucleic acids from foreign nucleic acids (see section 3.2.3).

### 3.2.1 Recognition of nucleic acids

Nucleic acids were discovered in 1869 by Friedrich Miescher<sup>93</sup>, and they are essential to all known forms of life. Two kinds of nucleic acids are known, RNA and DNA, both of which are composed of nucleotides (nucleobases, sugar moieties, and phosphate groups).

RNA is involved in several processes, such as encoding, and the expression and regulation of genes. The nucleobase in RNA nucleotides can be adenine, cytosine, guanine, or uracil. In addition to the identity of the nucleobase, RNA nucleotides can also differ in modifications of the sugar moiety or of the nucleobase. Different types of RNA are known. In a cell, mRNA, tRNA, rRNA, and different regulatory RNAs such as miRNA can be found. Most of them exist as single-stranded RNA, but some, e.g. tRNAs, have secondary and tertiary structure. Moreover, the RNA viruses have a genome of single-stranded or double-stranded RNA.

Recognition of "self" nucleic acids can contribute to autoimmune and auto-inflammatory disease (see section 3.2.3). Even so, the benefit of recognising foreign nucleic acid from invading pathogens appears to outweigh the negative consequences of possibly reacting to "self" nucleic acids.

### 3.2.2 Recognition of RNA by TLRs

Nucleic acid-sensing TLRs are a distinct subfamily of receptors that are uniquely located in endosomes<sup>94</sup>. Their localisation in endosomes enables recognition during viral uptake, for example, because their contact with host-derived RNA is limited under physiological conditions. All nucleic acid-sensing TLRs make use of similar signalling cascades and intracellular trafficking<sup>95</sup>. One group induced germline mutation using *N*-ethyl-*N*-nitrosourea

in a mouse model. It resulted in a phenotype failing to sense nucleic acids via TLR3, TLR7 and TLR9, all nucleic acid sensors identified at this time. The mutation was found to be due to a missense allele of *unc93b1*, encoding the 12-membrane-spanning protein Unc93b1<sup>95</sup>. Unc93b1 is a conserved protein of the endoplasmic reticulum (ER) that is crucial for delivering TLRs from the ER to the endosome<sup>95</sup>. All nucleic acid-sensing TLRs identified to date are Unc93b1-dependent, and are therefore located in the endosome. Moreover, trafficking of endosomal TLRs is affected by the ER chaperone gp96<sup>96,97</sup>. The recognition of nucleic acids by endosomal TLRs may be aided by accessory proteins such as high mobility group box (HMGB). HMGB1, HMGB2, and HMGB3 bind to immunogenic RNA and DNA and thereby contribute to the activation of TLRs. HMGBs increase uptake of nucleic acids and function as universal nucleic acid labels<sup>98</sup>.

### 3.2.2.1 RNA recognition by TLR3

TLR3 is expressed in macrophages and several subsets of DCs, but not in plasmacytoid DCs (pDCs) and monocytes<sup>99-102</sup>. It is like all other nucleic acid-sensing TLRs located in the endosome. TLR3 is stimulated by double-stranded RNA (dsRNA)<sup>37</sup>. The detection of RNA is sequence-independent and controlled by its secondary structure<sup>37</sup>. TLR3 was the first TLR for which a crystal structure of the ligand-bound ectodomain was described<sup>34</sup>. The dsRNA helix binds on two sides of the ectodomain, located at opposite ends of the TLR3 horseshoe<sup>103,40,34</sup>. To be recognised, a critical length of 40 base pairs (bp) is needed<sup>34,104</sup>. This enables binding of RNA at two TLR3 ectodomains, which stabilise and coordinate the dimer by intermolecular contact. The RNA binding sites of TLR3 function at pH levels below 6.5 - a hallmark of the endosomal environment - due to protonation of histidine residues<sup>34,105,106</sup>. Thus, positively charged amino acids are able to bind negatively charged RNA. The critical length of 40 bp also prevents small cellular RNAs such as tRNA and miRNA from being sensed. Typically, TLR3-bound dsRNA occurs as an intermediate during viral replication. Furthermore, mammalian RNA has been postulated to stimulate TLR3 in the presence of necrotic debris from neighbouring cells<sup>107</sup>. The importance of recognition of viral infection by TLR3 is shown by the fact that immune responses to West Nile virus, murine cytomegalovirus, and Semliki forest virus have been found to be limited in TLR3-deficient mice<sup>108,109,110</sup>.

As a surrogate for natural dsRNA, many studies have used poly I:C, which is also able to stimulate RIG-I and MDA5 in the cytosol<sup>111</sup>.

### 3.2.2.2 Recognition of RNA by TLR7 and TLR8

TLR7 and TLR8 are two receptors that recognise single-stranded RNA (ssRNA) <sup>33,32,31</sup>. Comparison of the sequences of all vertebrate TLRs indicated that TLR7 and TLR8 are most closely related; the next closely related is TLR9, then TLR3 <sup>112</sup>.

In the murine system, the functional competence of TLR8 is unclear. The *tlr8* gene is transcribed to mRNA, but TLR7-deficient mice are unable to respond to RNA in viral infections. It is therefore unclear whether the TLR8 receptor is functional. Furthermore, a five-amino acid region has been identified in all species with a functional TLR8 receptor and this region is missing in mice. Thus, the lack of this sequence may be responsible for the inactivity of the receptor <sup>113</sup>. Another hypothesis is that TLR8 in mice might have an inhibitory function and modulate the immune response of other TLRs such as TLR7 or TLR9. Indeed, TLR8-deficient mice show higher expression of TLR7 and increased responses to TLR7 ligands in DCs, resulting in increased susceptibility to autoimmune diseases <sup>114</sup>.

In the human system, TLR7 is expressed exclusively in pDCs and B cells, and TLR8 is expressed in macrophages and conventional DCs. In the murine system, TLR7 is more broadly expressed and can be found in macrophages and several DC subsets <sup>99,100</sup>. The differential expression in the human system may explain the individual cytokine profiles of the two receptors <sup>115</sup>. The overlapping expression in murine cells possibly neutralizes the effect of an inactive TLR8 <sup>116</sup>.

TLR7 and TLR8 share specificity for ssRNAs and small synthetic molecules belonging to the imidazoquinoline group <sup>33,30,32,31</sup>. In the human system, TLR7 is important for release of interferon-alpha (IFN- $\alpha$ ) from pDCs and it preferentially recognises guanosine-/uridine-rich sequences <sup>32,31,115</sup>. Adenosine-/uridine-rich sequences appear to be preferred by TLR8, which induces production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and IL12 <sup>115</sup>. The minimum length of RNA for efficient stimulation is debated, but 21 nucleotides appear to be sufficient <sup>32,117,118</sup>. The preference for specific nucleotide sequences may be related to the RNA sequence composition of certain viruses, e.g. influenza A virus, human immunodeficiency virus, and vesicular stomatitis virus <sup>33,32,31,119</sup>. Also, TLR7 plays an important role in sensing bacterial infections - as reported for group B streptococci <sup>116</sup>. In addition to Unc93b1, trafficking of TLR7 in pDCs is dependent on recruitment of AP-3 and LC-3. They are responsible for localisation of the receptor in endosomal compartments specialised in type-I IFN induction <sup>120,121</sup>.



As in all other TLRs, the ectodomains of TLR7 and TLR8 form a horseshoe structure. For TLR8, a crystal structure of the ectodomain with and without bound 3-diamino-furo[2,3-c]pyridine (a TLR8 ligand) has been published. Upon ligand binding, TLR8 is cleaved but the two halves remain associated and mediate dimerisation <sup>122</sup>.

TLR7 and TLR8 not only sense ssRNA; structural complexity is also important for recognition via TLR7. One kind of RNA that forms complex structures and that is recognised by TLR7 or TLR8 is tRNA. A tRNA molecule has one RNA strand with intramolecular base pairing and a complex three-dimensional structure. So ssRNA cannot be the only feature of RNA recognised by TLR7 and TLR8 <sup>123,124</sup>.

Besides RNA, a number of small molecules called imidazoquinolines are capable of activating TLR7, TLR8, or both <sup>30,17,125</sup>. Some of them are used in clinical therapeutics and they are often described as nucleoside analogues. The most important representatives of this group are Imiquimod and Resiquimod. Imiquimod (also known as R837) is a human and murine TLR7 agonist and it is used for treatment of genital warts and malignant skin tumours <sup>126</sup>. Resiquimod (also known as R848) is recognised by TLR7 and TLR8 and is used as a drug for the treatment of skin lesions caused by viruses such as herpes simplex virus <sup>127,128</sup>. It is also used as an adjuvant in vaccines <sup>129</sup>. Both ssRNA oligoribonucleotides and imidazoquinolines are recognised by the ectodomains of TLR7 and TLR8. The small size of imidazoquinolines indicates that their mode of recognition by TLR7 and TLR8 differs from that of oligoribonucleotides <sup>130</sup>. Studies in monocyte-derived DCs have suggested that there are differences in phosphorylation and transcriptional events activated by oligoribonucleotides and imidazoquinolines downstream of TLR8. In TLR7 and TLR8, overlapping and non-overlapping binding sites for RNA and R848 have been found, indicating that imidazoquinolines require multiple or extended sites for binding to the receptors <sup>130</sup>. The latter study suggested that the different classes of ligand (imidazoquinolines and oligoribonucleotides) can have different beneficial and harmful cellular effects <sup>130</sup>.

### 3.2.2.3 Recognition of RNA by TLR13

TLR13 is an endosomal receptor that is only expressed in mice and rats. The *tlr13* gene is located on the X chromosome, and its ligand was unknown until two groups independently reported in 2012 that bacterial RNA (bRNA) is the ligand for TLR13.

Hidmark et al. found reduced cytokine production in DCs after bRNA stimulation when TLR13 was knocked down by siRNA <sup>35</sup>. Furthermore, they showed activation of NFκB in

TLR13-transfected Chinese hamster ovary (CHO) cells - by bRNA stimulation and addition of *Streptococcus pyogenes* - in an RNA-dependent manner.

Oldenburg et al. observed an activation of murine DCs by *Staphylococcus aureus* (*S. aureus*) in a TLR2-, TLR3-, TLR4-, TLR7-, and TLR9-independent manner <sup>36</sup>. Furthermore, the activation was MyD88-dependant, suggesting involvement of a TLR. Further experiments with different DC subsets of several knock-out mice suggested a role of TLR13 in recognition of *S. aureus*. To find the exact ligand for TLR13, *S. aureus* preparations were treated enzymatically and the signal was abrogated after use of ssRNA-specific RNase. Fractionation of RNA identified 23S rRNA as the TLR13 ligand. The stimulation of TLR13 by 23S rRNA could be abrogated after growing *S. aureus* in the presence of erythromycin. In most cases, resistance to erythromycin is mediated by erythromycin resistance methylases (erm) that methylate a highly conserved adenosine at position 2058 (*E. coli* nomenclature), where the macrolides bind. This position is located in the sequence 'CGGAAAGACC', which has been identified as the stimulatory sequence of TLR13. Thus, it is a nucleic acid-sensing receptor with (as far as is known) strict sequence specificity. The sequence is highly conserved among gram-positive and gram-negative bacteria. Methylation or mutation of adenosine 2058 leads to a complete loss of TLR13 stimulatory capacity <sup>36</sup>.

**Table 3-1: Different RNA-sensing receptors with their respective ligands and recognition principles**

| Receptor | Ligand(s)                      | Recognition principle                                   |
|----------|--------------------------------|---|
| TLR3     | dsRNA, poly I:C                | double-stranded, at least 40 bp                         |
| TLR7     | ssRNA, siRNA, tRNA, R837, R848 | GU-rich RNA sequences                                   |
| TLR8     | ssRNA, siRNA, tRNA (?), R848   | AU-rich sequences                                       |
| TLR13    | bRNA                           | sequence-specific and modification-dependent            |
| RIG-I    | viral RNA, poly I:C            | double-stranded, up to 2 kbp in length, 5'-triphosphate |
| MDA5     | viral RNA, poly I:C            | double-stranded, at least 2 kbp                         |
| NLRP3    | bRNA                           | unknown   |
| NOD2     | ssRNA                          | unknown   |

Of all the nucleic acid-sensing TLRs, TLR13 has the highest sequence specificity identified so far; only TLR9 has been shown to recognise DNA with similar sequence specificity for CpG motifs. TLR13 recognises a defined sequence in 23S rRNA, a ligand only found as non-self RNA. All the RNA-sensing receptors with their exact ligands and recognition principles are listed in Table 3-1.

### **3.3 Discrimination of self RNA and foreign RNA**

#### **3.3.1 The danger-associated molecular pattern theory**

The immune system is not only activated by PAMPs; it is also able to recognise so-called danger- or damage-associated molecular pattern (DAMPs). Since the 1950s, the theory of non-self recognition predominated. In the 1990s, Matzinger published the “danger theory”, discussing that non-self structures can be tolerated if they are not dangerous. Examples include commensal bacteria and the fetus<sup>131–140</sup>. On the other hand, even self-compounds must be recognised if they are dangerous, for example during cellular stress or infection. Different host molecules have been identified that are capable of stimulating the immune system. In many cases, these molecules are secreted actively by infected or inflammatory cells, or they are released passively by necrotic cells. They are not released by apoptotic cells under physiological conditions.

For example, heat shock proteins (HSPs) are a highly conserved protein family, and they play an important role as DAMPs<sup>138,141,142</sup>. They are involved in folding, protection, and transport of proteins. HSPs can bind antigens and activate APCs. Other examples of DAMPs are uric acid, HMGB1, S100 proteins, and high concentrations of ATP<sup>143–147</sup>.

Nucleic acids are also a kind of DAMP, as they are released by dying cells and recognised by the immune system. Thus, in many cases the immune system has to distinguish between self RNA and non-self RNA. All nucleic acid-sensing receptors are able to sense mammalian RNA under pathophysiological conditions<sup>148</sup>. If discrimination fails, and self RNA or even other molecules are recognised in the absence of danger or damage, autoinflammation and autoimmune diseases can occur.

### 3.3.2 Autoimmune diseases

Autoimmune and auto-inflammatory diseases occur when the immune system attacks and destroys healthy tissue. It results from a misled immune activation. Nucleic acid-sensing TLRs especially - such as TLR7, TLR8, and TLR9 - have been reported to be involved in autoimmune diseases like systemic lupus erythematoses (SLE) and psoriasis<sup>149-154</sup>. SLE can affect any part of the body. It attacks the body's cells and tissues, leading to inflammation and tissue damage<sup>155</sup>. Psoriasis is an immune-mediated skin disease characterised by red, scaly patches, papules, and plaques<sup>156</sup>. The exact mechanisms that lead to autoimmune diseases are still unclear, but many of the components involved have been identified. IgG antibodies known as rheumatoid factors are produced by B cells after dual stimulation of antigen receptors and TLR9<sup>157,158</sup>. When the uptake and the delivery of self DNA/chromatin complexes are enhanced, the TLR-mediated discrimination of self from foreign breaks down and self DNA induces stimulation. For SLE, it has been proposed that there is a TLR-independent stage and a TLR-dependant stage. First, a TLR-independent uptake of debris derived from self cells by DCs takes place, followed by TLR-dependant recognition of the ingested nucleic acids. Both of these parts are driven by type-I interferons. Studies with ectopic expression of TLR9 at the cell surface instead of in endosomes, resulting in recognition of self DNA, have indicated the importance of spatial restriction<sup>159</sup>. Similar results have been shown for RNA-containing immune complexes, triggering B cell receptors and TLR7<sup>160</sup>. The uptake of nucleic acid/immunoglobulin complexes into pDCs is mediated by the low-affinity Fc receptor (FcγRIIA) and leads to TLR9 activation<sup>154</sup>. The delivery of nucleic acids can also be mediated by the anti-microbial peptide LL37. It enhances the transport of aggregated DNA into early endosomes, leading to TLR9 stimulation. Thus, LL37 converts self DNA to stimulatory DNA. This complex is resistant to DNase and plays an important role in pDC activation in psoriasis. It has been suggested that similar mechanisms are important for triggering of TLR7 with self RNA<sup>161</sup>. Recognition of nucleic acids by TLRs may play a role in activation of pDCs and B cells, leading to production of autoantibodies against DNA and RNA - as observed in SLE<sup>162</sup>. Hence, the duplication of *tlr7* promotes autoimmune diseases, pDC activation, enhanced type-I interferon production, and auto-antibody formation in mice<sup>163,164</sup>. Furthermore, TLR7 deficiency leads to reduced sensitivity to autoimmune diseases and to lower serum levels of autoantibodies<sup>165,166</sup>.

TLR3 is associated with autoimmune liver damage and rheumatoid arthritis<sup>167-169</sup>. Incubation of TLR3-expressing fibroblasts with necrotic synovial fluid cells from patients suffering from rheumatoid arthritis leads to increased chemokine and cytokine levels<sup>170,171</sup>.

Endosomal TLRs not only have a role in the direct recognition in autoimmunity. Studies with Unc93b1-deficient patients have shown defects in B cell tolerance, leading to accumulation of autoreactive B cells - thus indicating that TLRs have a role in B cell maturation<sup>172</sup>.

These reports indicate that self-tolerance is important for nucleic acid-recognising TLRs and that discrimination of self from foreign is not specific to microbes, thus making spatial restriction by nucleic acid-sensing TLRs even more important.

The spatial restriction prevents the conjunction of receptor and self-nucleic acid under physiological conditions<sup>31,32,94,159</sup>. Furthermore, on the way to the endosomes, TLR9 is not functionally active. It has to be cleaved by a pH-dependent protease after endolysosome formation to be able to recognise RNA<sup>173,174</sup>. This prevents recognition of cytosolic RNA during translocation<sup>175</sup>. It has also been shown for TLR7<sup>176</sup> and may be true for other TLRs.

### 3.3.3 RNA modifications

Incorporation of modifications is an important tool to mark RNA as self, and thus prevent it from immune recognition. The importance of RNA modification depends on the TLR recognising the specific RNA.

TLR3 recognises dsRNA with a minimum length of 40 bp. In an uninfected cell, only short, structured RNA such as tRNA and miRNA is present, but no RNA with a double-stranded motif of 40 bp. dsRNA of this minimum length is only present in virally infected cells, where the dsRNA is either derived from viral genomes or from replication intermediates<sup>161</sup>.

For TLR13, the discrimination is brought about by the fact that the receptor recognises a specific sequence that is only present in prokaryotic rRNA, but not in eukaryotic ones<sup>36</sup>.

TLR7 and TLR8 recognise ssRNA with a minimum length of 20 nucleotides. This length is found in mammalian cells, making other characteristics important for discrimination. While viral RNA uses the cellular machinery for replication of RNA, processing of bacterial RNA differs from that of mammalian RNA. Efficient identification of mammalian RNA is possible because of the cap structure of mRNA, polyadenylation, and post-transcriptionally introduced nucleotide modifications<sup>161</sup>. Secretion of IL12 by human monocyte-derived DCs is enhanced by stimulation with bRNA and low upon stimulation with mammalian RNA<sup>177</sup>.

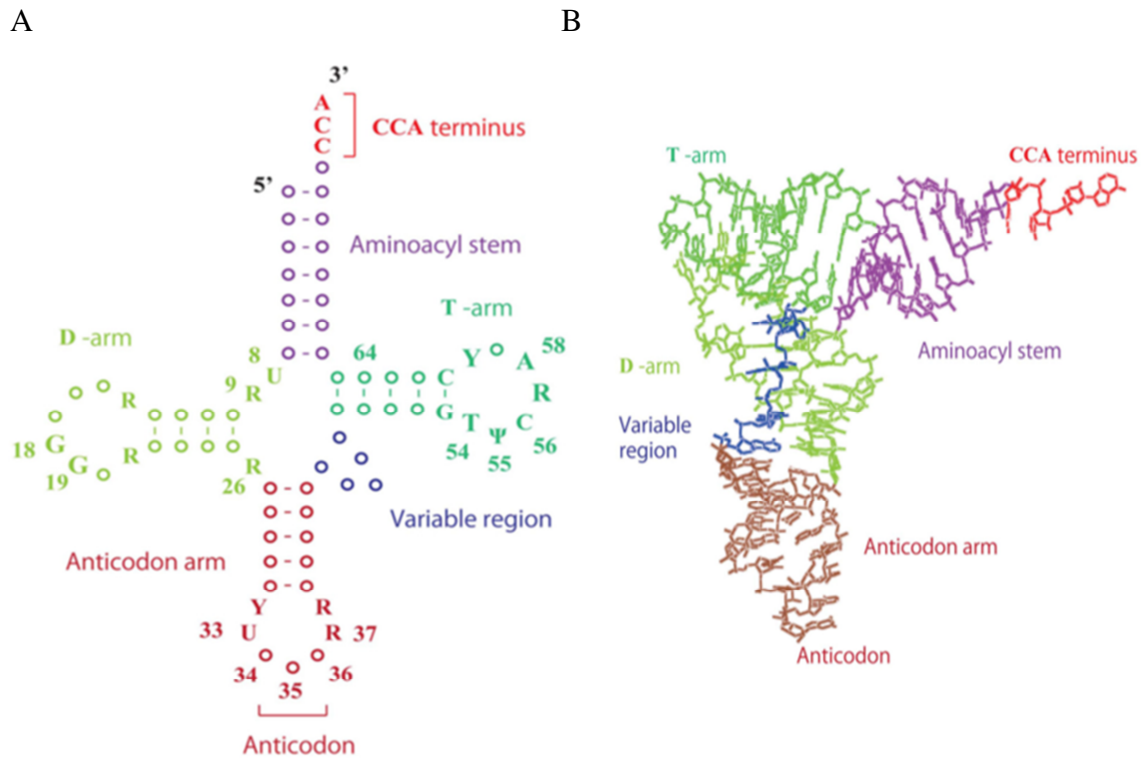
*In vitro*-transcribed mRNA with no polyadenylation is again stimulatory, while stimulation after enzymatic 3'-polyadenylation abrogates cytokine secretion. This shows the important influence of polyadenylation in the identification of mammalian RNA. Also, post-transcriptionally introduced nucleotide modifications play an important role in discrimination. They are abundant in eukaryotic RNAs and are much less common in bacterial and mitochondrial RNA<sup>178</sup>. The lower frequency of modifications in mitochondrial RNA is due to the bacteria-like processing of RNA in the mitochondria as a result of their bacterial origin. In human rRNA, ten times more pseudouridines ( $\Psi$ ) and 25 times more 2'-O-methylated nucleosides are present than in bacterial rRNA. tRNA is the most commonly modified RNA; in mammals, up to 25% of all nucleosides in tRNA are modified<sup>179</sup>.

Modifications can also be found in the internal region of viral RNAs, for example in influenza virus, adenovirus, and herpes simplex virus. Often the viral mRNA is more frequently modified than the cellular mRNA, due to methyltransferases encoded by the virus. Karikó et al. observed in 2005 that there was a correlation between the number of modifications and potency of stimulation<sup>178</sup>. Their studies showed that the lower the number of modifications in the RNA, the higher was their immunostimulatory capacity. Further investigations identified different modifications that were able to abrogate stimulation of immune cells. *In vitro*-transcribed RNA with N6-methyladenosine ( $m^6A$ ), 5-methylcytidine ( $m^5C$ ), 5-methyluridine ( $m^5U$ ), 2-thiouridine ( $s^2U$ ), and pseudouridine ( $\Psi$ ) circumvents recognition by TLR7 and TLR8<sup>180,181</sup>. Thus, the amount and kinds of modifications appear to be important factors in discrimination between self RNA and foreign RNA.

### 3.3.3.1 Naturally occurring modifications in tRNAs

The RNA population of a cell consists of 80–90% rRNA and around 10% tRNA. This suggests that rRNA would possibly be important for recognition by immune receptors. However, rRNA is mostly present in ribosomes, and is therefore covered by proteins. Thus, tRNA is probably the most free RNA and is therefore the most likely to be recognised<sup>161</sup>. As previously mentioned, in mammalian tRNA up to 25% of the nucleotides are modified and it is therefore the most abundantly modified RNA<sup>179</sup>. The different tRNA modifications are linked to the different steps of protein synthesis<sup>182</sup>. Unmodified tRNAs fail to produce proper proteins<sup>182</sup>. The three loop regions are the most frequently modified parts of tRNA. The clover-leaf structure of tRNA is shown in Figure 3-2A, and the numbers indicate the

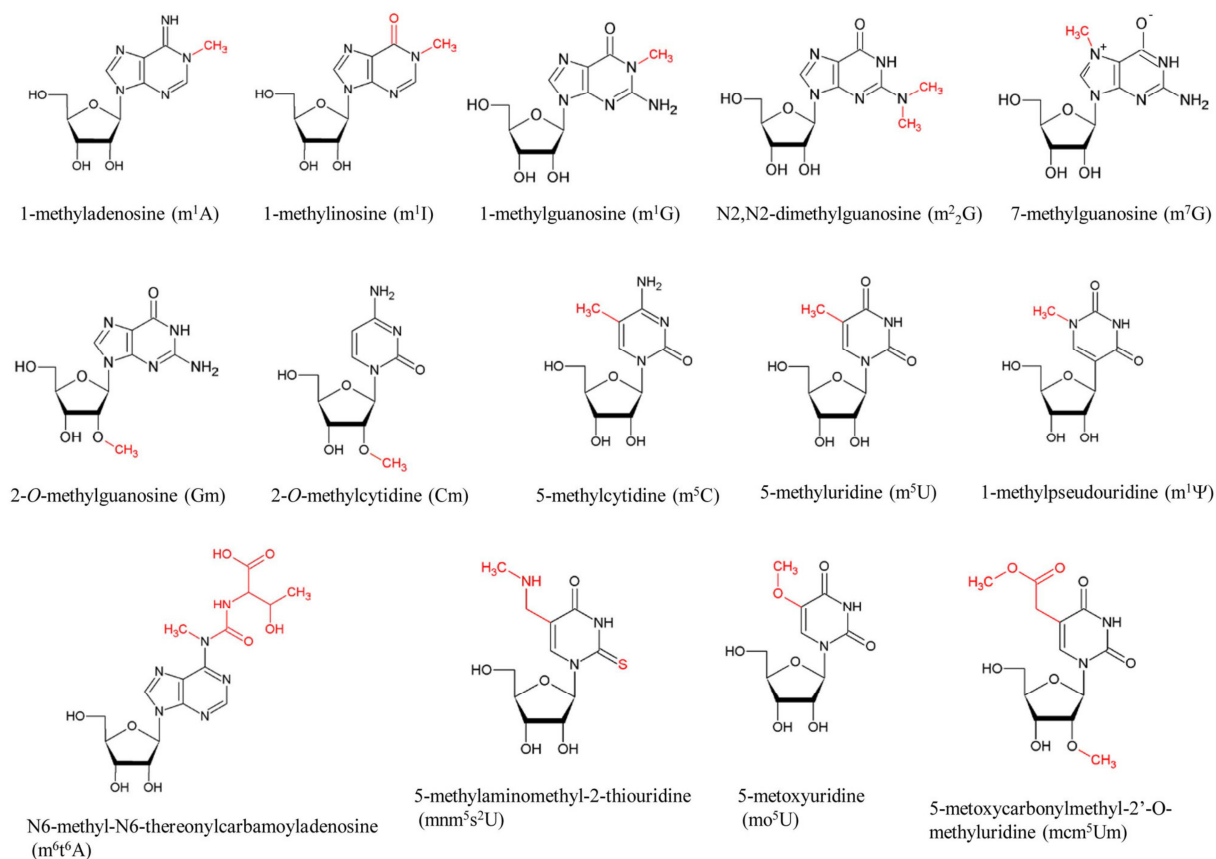
positions of modified nucleotides<sup>182</sup>. Panel B shows the three-dimensional structure of tRNA. The D-arm and the T-arm interact resulting, in an L-shaped structure.



**Fig. 3-2: The structure of tRNA.** (A) The clover-leaf structure of a model tRNA. The numbers show the positions of the nucleosides. (B) The L-shaped tRNA structure, in which the D- and T-arms interact by tertiary base pairing.<sup>182</sup>

Methylations are important modifications in tRNAs; they are involved in stability and recognition. Methylation of tRNAs is mediated by different sequence- and position-specific tRNA methyltransferases. For example in *E. coli*, uracil-5-methyltransferase (trmA) is responsible for methylation of the uridine at position 54 (m<sup>5</sup>U<sub>54</sub>) of tRNA and guanosine-7-methyltransferase (trmB) methylates the guanosine located at position 46 (m<sup>7</sup>G<sub>46</sub>) of tRNA<sup>183,184</sup>. Gm18-2'O-methyltransferase (trmH) methylates the guanosine at position 18. This nucleoside at position 18 is highly conserved in the D-loop of tRNAs<sup>185</sup>. It interacts with the conserved pseudouridine at position 55 in the T-loop. Together, they stabilise the L-shaped three-dimensional structure of tRNA<sup>124</sup>. The structures of typical methylated nucleosides are shown in Figure 3-3<sup>182</sup>.

Most of the studies on modified RNAs have been performed with synthetic RNAs. Different modifications were incorporated into nucleosides with the same nucleobase; for example, all adenosines were methylated. In addition, the modifications were not tested in the natural environment - i.e. sequence context, structure, and origin were not taken into account<sup>178,180,181,186-188</sup>.



**Fig. 3-3: Typical methylated nucleosides in tRNAs.** The modifications are shown in red. The abbreviations for the modified nucleosides are shown in parentheses.<sup>182</sup>

### 3.3.3.2 2'-O-methylation of RNA

2'-O-methylation of ribose has been found as a modification in eukaryotic RNA, efficiently preventing TLR activation and resulting in an immunosilent RNA<sup>189</sup>. Several studies have found a suppressive effect of 2'-O-methylation on TLR7 activation in human and murine pDCs and monocytes<sup>187,189–191</sup>; thus, 2'-O-methylated RNA inhibits stimulation by simultaneously added stimulatory RNA. Thus, this RNA modification is immunoinhibitory. 2'-O-methylation is much more abundant in eukaryotic RNA than in bacterial and mitochondrial RNA. 2'-O-methylations have been found in mRNA, rRNA, tRNA, and several other non-coding RNAs<sup>192–194</sup>.

To test modifications as mechanisms of discrimination between RNAs in their natural context, Gehrig et al. isolated and analysed native tRNAs for their immunostimulatory capacity<sup>123</sup>. Preparations of single tRNAs from *E. coli* (containing naturally occurring modifications) were compared with corresponding *in vitro* transcripts (without modifications) regarding their immune stimulation. Surprisingly, the tRNA for tyrosine (tRNA<sup>Tyr</sup>) lacked immune stimulation whereas the corresponding *in vitro* transcript was able to induce type-I interferon production



in human peripheral blood mononuclear cells (PBMCs). Thus, the observed difference could only have been due to a modification. Furthermore, 2'O-methylation at G18 was identified as being necessary and sufficient for abrogation of stimulation<sup>123</sup>.

### 3.4 Objectives of this work

As outlined in the previous sections, microbial RNA has an important role as a target for detection of invading pathogens. Even so, under pathophysiological conditions self RNA can also be recognised, resulting in autoimmune and auto-inflammatory diseases. To distinguish between self and non-self RNA, naturally occurring RNA modifications can be used. Mammalian RNA - especially tRNA - is abundantly modified, whereas bacterial RNA contains fewer and different modifications. Interestingly, modifications introduced into mammalian RNA can silence or even inhibit recognition by and stimulation of the innate immune response. Most of the studies performed so far have analysed the effects of modifications in synthetic RNA oligonucleotides or in *in vitro* transcripts. The aim of this thesis was to determine the properties of one RNA modification in its natural context. The intention was to study the immunological effects of 2'O-methylation at position G18 of *E. coli* tRNA<sup>Tyr</sup>.

The central hypothesis of the work for this thesis was that 2'O-methylation - when incorporated into the natural sequence of a small oligoribonucleotide - would be capable of suppressing immunostimulation. It was predicted that 2'O-methylated RNA would block the otherwise activated TLR7.

We therefore investigated whether 2'O-methylated RNA can suppress immunostimulation by tRNA and by total RNA preparations from different bacterial species. If RNA preparations could be identified that (by themselves) did not show induction of immunostimulation, the preparations would be screened for additional modifications. To determine whether the inhibitory effect was specific for IFN- $\alpha$  production through TLR activation, the secretion of other cytokines was analysed. If any influence on other cytokines was observed, the relevant cell type and receptor would be identified. To determine the mechanism of inhibition, the signalling pathways of TLRs would have to be studied.

Furthermore, the influence of the surrounding sequence next to the 2'O-methylated G18 for immunoinhibition needed to be studied. Thus, nucleobases in 2'O-methylated tRNAs would be mutated and the resulting tRNAs tested for immunostimulation. We believed that a specific "inhibitory" sequence motif might exist.

## 4 MATERIALS AND METHODS

### 4.1 Materials

#### 4.1.1 Cells

##### 4.1.1.1 Mouse strains

Primary murine cells were isolated from C57BL/6 mice purchased from Charles River Laboratories (Willmington, MA, USA) and bred under specific pathogen free (SPF) conditions. Breeding and monthly control of infection were performed according to the list of GV-SOLAS 1995. Killing and dissection were approved by the regional commission in Tübingen. Experiments were properly recorded and reported to Tübingen.

##### 4.1.1.2 Eukaryotic cells

All eukaryotic cells were cultured at 37 °C, 5% CO<sub>2</sub> in a humidified environment. The composition of the medium is described below.

**Murine BMDCs:** Murine immune cells were generated from bone marrow cells to macrophages and dendritic cells. The bone marrow of tibiae and femur were used as described in 4.2.1.2 (Generation of murine primary bone marrow-derived dendritic cells).

**Human PBMCs:** Human primary immune cells were isolated from blood of healthy donors as described in 4.2.1 (Isolation of human peripheral blood mononuclear cells).

**293XL-hTLR7-HA:** HEK293XL cell line expressing HA-tagged human TLR7 gene, InvivoGen, San Diego, USA

##### 4.1.1.3 Media composition

**Inactivation of fetal calf serum (FCS):** Sterile FCS was heat inactivated at 56 °C for 1 h to inactivate contained complement proteins. Afterwards it was decanted into aliquots and stored at -20 °C.

**Differentiation medium for BMDC:** RPMI 1640 was supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) Penicillin/Streptomycin and 50 mM 2-Mercaptoethanol.

**Stimulation medium BMDC:** RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS

**Stimulation medium PBMCs:** RPMI supplemented with 10% (v/v) heat inactivated FCS

**Medium for HEK cells:** DMEM supplemented with 10% (v/v) heat inactivated FCS, for HEK-TLR7HA further complemented with 10 µg/ml Blasticidin

#### 4.1.2 Instrumentation

All instruments used through this study are listed in Table 4-1.

**Table 4-1: All instruments used in the study**

|   |  |
|---|--|
| AutoMACS  | Miltenyi Biotech, Bergisch Gladbach, Germany   |
| Balance   | EW600-2M, Kern & Sohn GmbH, Balingen, Germany  |
| Blotting chamber  | Trans-blot <sup>®</sup> SD Semi-dry transfer cell, Bio-Rad, München  |
| Centrifuges   | Biofuge stratos, Multifuge 3S-R, Heraeus Instrumens, Hanau<br>Biofuge fresco, Heraeus Instrumens, Hanau, Germany<br>Biofuge pico, Heraeus Instrumens, Hanau, Germany |
| Confocal microscope                                       | Leica TCS SP5, Leica Microsystems, Mannheim  |
| Counting chamber  | Neubauer 0.00025 mm <sup>2</sup> /0.1 mm, Brand GmbH, Schwerin   |
| Electrophoresis chamber                                   | Perfect Blue Mini ExM, Gelsize 14.4 x 10.2 cm, Peqlab, Erlangen  |
| Flow Cytometry  | BD FACS Canto (2-laser, 6 colour), BD Bioscience, Heidelberg   |
| Gel Documentation-System<br>(Chemiluminescence detection) | CN-3000.WL, Peqlab, Erlangen   |
| Heating block   | Thermomixer comfort, Eppendorf AG, Hamburg   |
| Incubator   | BBD6226, Heraeus Instrumens, Hanau   |
| Magnetic shaking device                                   | IKA <sup>®</sup> RCT basic, IKA-Labortechnik, Staufen i. Br.   |
| Microtiterplate Photometer                                | SUNRISE Absorbance Reader, Tecan, Salzburg, Austria  |
| Orbital Shaker  | Mini Rocker MR-1, Peqlab, Erlangen<br>Rocky <sup>®</sup> 3D, Labortechnik Fröbel GmbH, Lindau<br>RS-24, Lab4You GmbH, Berlin   |
| pH-Meter  | Seven Easy, Mettler Toledo, Gießen   |
| Power Supply  | Power Supply 1000/500, BioRad, München   |

|                            |   |
|----------------------------|---|
| Reflected light microscope | Leica DMLS, Leica Microsystems GmbH, Wetzlar  |
| Safety cabinet             | Herasafe, Class II, Heraeus Instrumens, Hanau   |
| SDS-PAGE system            | perfectBlue™ Twi S, Peqlab, Erlangen  |
| Thermocycler               | Primus 25 advanced®, Peqlab, Erlagen<br>Primus 96 advanced® gradient, Peqlab, Erlangen<br>StepOnePlus Real-Time PCR System, Applied Biosystems, Darmstadt |
| Spectrophotometer          | NanoDrop® ND-1000 Spectrophotometer, Peqlab, Erlangen   |
| UV platform                | ECX-26M, Peqlab, Erlangen   |
| Vortexer                   | MS 1, IKA® Inc., Wilmington, USA  |

### 4.1.3 Consumables

All consumables used through the study are listed in Table 4-2.

**Table 4-2: List of all used consumables with suppliers**

|                                   |  |
|-----------------------------------|--|
| Blot membrane, Nitrocellulose     | Immobilon-P Transfer, 0.45 µm pore size, Millipore Billerica, USA  |
| Blotting paper                    | Whatman® GB003, Whatman GmbH, Dassel   |
| Caps                              | Eppendorf-caps 0.5 ml, 1.5 ml, 2 ml; Eppendorf AG, Hamburg   |
| Cell culture bottles              | Cellstar® 25cm <sup>2</sup> / 75 cm <sup>2</sup> / 175 cm <sup>2</sup> , Greiner Bio-One GmbH, Frickenhausen |
| Cell culture plates (flat bottom) | Cellstar® 6- / 24- / 96- well plates, Greiner Bio-One GmbH, Frickenhausen                                    |
| Cell culture dishes               | 58 cm <sup>2</sup> ; 145 cm <sup>2</sup> , Nunc GmbH & Co. KG, Wiesbaden                                     |
| Cell scraper                      | Disposable Cell Scraper, Greiner Bio-One GmbH, Frickenhausen   |
| Cryo tubes                        | Nunc GmbH & Co. KG, Wiesbaden  |
| Gloves                            | Touch N Tuff Disposable Nitrile Gloves, Ansell, Brussels, Belgium  |
| ELISA plates                      | 96 well half area plates, Greiner Bio-One GmbH, Frickenhausen  |
| FACS tubes                        | BD Falcon™ 5 ml, BD Bioscience, Heidelberg   |

|              |  |
|--------------|--|
| PCR plates   | MicroAmp Fast 96-well reaction plate (0.1 ml), Applied Bioscience, Darmstadt               |
| PCR tubes    | PP-PCR-tubes, Nat. 0.2 ml, Greiner Bio-One GmbH, Frickenhausen                             |
| Petri dishes | 145 x 20 mm, Greiner Bio-One GmbH, Frickenhausen   |
| Pipet tips   | TipOne, Star Lab, Hamburg<br>Premium Line® Filter Tips, AHN Bioechnologie GmbH, Nordhausen |
| Syringe      | BD Discardit II 27 G, 5 ml, 10 ml and 50 ml, BD Bioscience, Heidelberg                     |
| Tubes        | Cellstar Tubes, 15 ml and 50 ml, Greiner Bio-One, Frickenhausen                            |

#### 4.1.4 Chemicals and reagents

All chemicals used for this study were purchased from Merck (Darmstadt) or Sigma-Aldrich (Taufkirchen) with a purity of “pro analysis”. All chemicals purchased from further companies listed in Table 4-3.

**Table 4-3: List of all used chemicals not provided by Merck or Sigma-Aldrich**

|  |   |
|--|---|
| Aceton                                     | Riedel-de Haën AG, Seelze                 |
| Acrylamid                                  | Roth, Karlsruhe                           |
| Agarose ultra pure                         | Gibco BRL, Life Technologies™, Eggenstein |
| Aqua ad iniectabilia (10 ml)               | Braun, Melsungen                          |
| Blasticidin                                | InvivoGen, Toulouse, France               |
| Dulbecco’s Modified aple Medium (DMEM, 1x) | Biochrom AG, Berlin                       |
| Ethanol                                    | Riedel-de Haën AG, Seelze                 |
| FACSClean™                                 | BD Bioscience, Heidelberg                 |
| FACSFlow™                                  | BD Bioscience, Heidelberg                 |
| Fetale Calf Serum (FCS)                    | Biowest, Nuaille, France                  |
| Isopropanol                                | Riedel-de Haën AG, Seelze                 |

|  |                                      |
|--|--------------------------------------|
| Lipofectamin™2000  | Invitrogen™, Karlsruhe               |
| Methanol   | Riedel-de Haën AG, Seelze            |
| Opti-MEM® I Reduced Serum Medium with GlutaMAX™ I        | Invitrogen™, Karlsruhe               |
| PBS (1x) for cell culture                                | PAA Laboratories, Pasching, Austria  |
| Penicillin/Streptomycin solution (100 x)                 | PAA Laboratories, Pasching, Austria  |
| Rotiphorese sequence gel system and sequence concentrate | Roth, Karlsruhe                      |
| RPMI 1640 Medium (1x)                                    | Biochrom AG, Berlin                  |
| Sodiumchloride (NaCl)                                    | AppliChem GmbH, Darmstadt            |
| Sodiumdodecylsulfate (SDS)                               | AppliChem GmbH, Darmstadt            |
| Sodiumfluoride (NaF)                                     | Roth, Karlsruhe                      |
| TRIzol® Reagent  | Ambion, life technologies, Darmstadt |

#### 4.1.5 Buffers and solutions

All buffers and solutions used in the study or listed with their composition in Table 4-4 to Table 4-7.

**Table 4-4: List of buffers and solutions used for western blot**

|                              |   |
|------------------------------|---|
| RIPA lysis buffer            | 50 mM Tris-HCL, pH 7.1; 1% (v/v) Igepal; 0.25% (v/v) sodium deoxycholat; 150 mM NaCl, 1mM EDTA<br>Add fresh before use: 1 µg/ml of Aprotinin, Leupepin, Pepstatin; 1 mM Na <sub>3</sub> VO <sub>4</sub> , NaF, PMSF |
| Acrylamid stock solution     | 30% (v/v) acrylamide and bisacrylamid solved 29:1   |
| SDS running uffer            | 25 mM Tris-base, pH 8.3; 192 mM Glycin; 0.1% (w/v) SDS  |
| SDS sample buffer (4x)       | 200 mM Tris-HCl, pH 6.8; 20% (v/v) β-Mercaptoethanol; 8% (w/v) SDS; 40% (v/v) Glycerol; 0.04% (w/v) Bromphenoleblue   |
| SDS blot buffer              | 25 mM Tris-base, pH 8.3; 192 mM Glycin; 20% Methanol  |
| SDS-stacking gel buffer (2x) | 250 mM Tris-HCl; 0.2% (w/v) SDS; degassed before use  |

|                                |  |
|--------------------------------|--|
| SDS separation gel buffer (3x) | 1.123 M Tris-HCl, pH 8.8; 0.3% (w/v) SDS; degassed before used |
| 10 x TBS                       | 100 mM Tris-HCl, pH 8.0; 1.5 M NaCl                            |
| Blocking Buffer                | 1 x TBS; 0.1% (v/v) Tween20; 5% (w/v) BSA                      |
| Wash Buffer                    | 1 x TBS; 0.1% (v/v) Tween20                                    |

**Table 4-5: List of medium, buffers and solutions used for cell culture**

|                         |   |
|-------------------------|---|
| Trypan blue solution    | 2 mg/ml trypan blue in 1 x PBS                                      |
| Penicillin/Streptomycin | 10000 U/ml penicillin G; 10 mg/ml streptomycin;<br>0.09% (w/v) NaCl |
| RPMI 1640 medium        | PAA laboratories, Egelsbach   |
| DMEM                    | PAA laboratories, Egelsbach   |

**Table 4-6: List of buffers and solutions used for ELISA**

|                         |   |
|-------------------------|---|
| Coating Buffer (pH 9.5) | 0.1 M Sodiumcarbonate: 8.4 g NaHCO <sub>3</sub> , 3.6 g Na <sub>2</sub> CO <sub>3</sub> ;<br>adjust to pH 9.5; ad 1000 ml H <sub>2</sub> O                  |
| Coating Buffer          | 0.2 M Sodiumcarbonate: 11.8 g Na <sub>2</sub> HPO <sub>4</sub> , 16.1 g NaH <sub>2</sub> PO <sub>4</sub> ;<br>adjust to pH 6.5; ad 1000 ml H <sub>2</sub> O |
| Blocking Buffer         | 1 x PBS; 10% (v/v) FCS  |
| Wash Buffer             | 1 x PBS; 0.05% (v/v) Tween20  |
| Assay Buffer            | 1 x PBS; 0,5% (w/v) BSA; 0,05% (v/v) Tween20  |

**Table 4-7: List of buffers used for immunoprecipitation**

|                |  |
|----------------|--|
| Lysis Buffer   | 136 mM NaCl, 20mM TrisHCl, 10% Glycerol, 2mM EDTA; adjust to pH7.4;<br>1% Triton X100<br>freshly added: 1 µg/ml of Aprotinin, Leupepin, Pepstatin; 1 mM Na <sub>3</sub> VO <sub>4</sub> , NaF,<br>PMSF   |
| Washing Buffer | 136 mM NaCl, 20mM TrisHCl, 10% Glycerol, 2mM EDTA; adjust to pH7.4;<br>0.1% Triton X100<br>freshly added: 1 µg/ml of Aprotinin, Leupepin, Pepstatin; 1 mM Na <sub>3</sub> VO <sub>4</sub> , NaF,<br>PMSF |

## 4.1.6 Biological reagents

### 4.1.6.1 Primer

All primers used through the study are listed in Table 4-9.

**Table 4-8: Primer sequences used for amplification transcripts**

| Gene              |    | Sequence 5'-3'          | Product size (cDNA) | Amplicon position         |
|-------------------|----|-------------------------|---------------------|---------------------------|
| h- $\beta$ -Actin | fw | AGAGCTACGAGCTGCCTGAC    | 183 bp              | 786-806                   |
|                   | rv | AGCACTGTGTTGGCGTACAG    |                     | 989-969                   |
| hIL1 $\beta$      | fw | GAGCAACAAGTGGTGTCTCC    | 140 bp              | 668-688                   |
|                   | rv | TCCAGCTGTAGAGTGGGCTTA   |                     | 807-787                   |
| hTNF              | fw | GCCCAGGCAGTCAGATCATCTTC | 181 bp              | 395-417                   |
|                   | rv | TGAGGTACAGGCCCTCTGATGG  |                     | 575-554                   |
| hIL6              | fw | GGATTCAATGAGGAGACTTGC   | 197 bp              | 772-792                   |
|                   | rv | GTTGGGTCAGGGGTGGTTAT    |                     | 968-949                   |
| hIL12p40          | fw | GCGGAGCTGCTACACTCTC     |                     |                           |
|                   | rv | CCATGACCTCCATGGGCAGAC   |                     |                           |
| unibac            | fw | AGAGTTTGATCMTGGCTCAG    | bacteria dependent  | 27-88 ( <i>E.coli</i> )   |
| 16SrRNA           | rv | CCGTCAATTCMTTTRAGTTT    |                     | 926-907 ( <i>E.coli</i> ) |

### 4.1.6.2 Markers

As marker for western blot analysis Page Ruler™ Plus Prestained Protein Ladder from Thermo Scientific, Karlsruhe was used.

### 4.1.6.3 Antibodies

All antibodies used for western blot analysis are listed in Table 4-9. The beads used for immunoprecipitation are listed in Table 4-10.



**Table 4-9: List of antibodies used for western blot analysis, all are from Cell Signaling, Frankfurt**

| Antigen                  | Species        | Phosphorylation sites | Final dilution |
|--------------------------|----------------|-----------------------|----------------|
| $\beta$ -actin           | rabbit         | --                    | 1:1000         |
| p-p44/42                 | rabbit         | Tyr204/Tyr202         | 1:1000         |
| I $\kappa$ B $\alpha$    | rabbit         |                       | 1:1000         |
| p- I $\kappa$ B $\alpha$ | rabbit         | Ser32                 | 1:1000         |
| p-JNK/SAPK               | rabbit         | Thr183/Thr185         | 1:1000         |
| p-p38                    | rabbit         | Thr180/Thr182         | 1:1000         |
| HA                       | rabbit         | --                    | 1:1000         |
| mouse IgG                | HRP-conjugated | --                    | 1:4000         |
| rabbit IgG               | HRP-conjugated | --                    | 1:4000         |

**Table 4-10: Substances used for immunoprecipitation**

| detected tag |                                 | company                      |
|--------------|---------------------------------|------------------------------|
| HA           | agarose conjugated antibody     | Sigma Aldrich, Taufkirchen   |
| Biotin       | agarose conjugated streptavidin | Thermo Scientific, Karlsruhe |

#### 4.1.6.4 Stimulatory agents and used RNA strands

All used RNA strands are listed in Table 4-11 to Table 4-13. All used TLR stimuli are listed in Table 4-14. The transfection agents used for transfection of RNA are listed in Table 4-15.

**Table 4-11: Sequences of RNA oligonucleotides used for experiments**

| name                      | sequence                            |
|---------------------------|-------------------------------------|
| inhibitory RNA (inh. RNA) | 5' GGUGGGGUUCCCGAGCGmGCCAAAGGGAG 3' |
| control RNA (ctr. RNA)    | 5' GGUGGGGUUCCCGAGCGGCCAAAGGGAG 3'  |

**Table 4-12: list of RNA oligonucleotides used to ligate tRNAs for motif studies**

| name         | sequence                                |
|--------------|---|
| fragment 1   | 5' GGUGGGGUUCCCGAGCGGCAAAG 3'           |
| fragment 1-1 | 5' GGUGGGGUUCCCGAGCGmGCAAAG 3'          |
| fragment 1-2 | 5' GGUGGGGUUCCCGAGCGGmCAAAG 3'          |
| fragment 1-3 | 5' GGUGGGGUUCCCGAGCGGmGCAAAG 3'         |
| fragment 1-4 | 5' GGUGGGGUUCCCGAGCGmCCCAAAG 3'         |
| fragment 1-5 | 5' GGUGGGGUUCCCGAGCGmUCCAAAG 3'         |
| fragment 1-6 | 5' GGUGGGGUUCCCGAGCGmACCAAAG 3'         |
| fragment 1-7 | 5' GGUGGGGUUCCCGAGCCmGCAAAG 3'          |
| fragment 1-8 | 5' GGUGGGGUUCCCGAGCUmGCAAAG 3'          |
| fragment 1-9 | 5' GGUGGGGUUCCCGAGCAmGCAAAG 3'          |
| fragment 2   | 5' GGAGCAGACUGUAAAUCUGCCGUCACAG 3'      |
| fragment 2-1 | 5' GGAGCAGACUGmUAAAUCUGCCGUCACAG 3'     |
| fragment 2-2 | 5' GGAGCAGACUGmAAAAUCUGCCGUCACAG 3'     |
| fragment 2-3 | 5' GGAGCAGACUGmGAAAUCUGCCGUCACAG 3'     |
| fragment 2-4 | 5' GGAGCAGACUGmCAAAUCUGCCGUCACAG 3'     |
| fragment 3   | 5' ACUUCGAAGGUUCGAAUCCUCCCCCACCACCA 3'  |
| fragment 3-1 | 5' ACUUCGAAGGUUCGmAAUCCUCCCCCACCACCA 3' |
| fragment 3-2 | 5' ACUUCGAAGGUUCGmGAUCCUCCCCCACCACCA 3' |
| fragment 3-3 | 5' ACUUCGAAGGUUCGmUAUCCUCCCCCACCACCA 3' |
| fragment 3-4 | 5' ACUUCGAAGGUUCGmCAUCCUCCCCCACCACCA 3' |

**Table 4-13: RNA oligonucleotides used for microscopy analysis, provided from IBA, Göttingen**

|    | unmodified RNA (5'→3')          | 2'O-methylated RNA (5'→3')          |
|----|---------------------------------|-------------------------------------|
| s  | GCAAGCUGACCCUGAAGUUCAU          | GCAAGCUGACCCUGAAGUUCAU              |
| as | (ATTO488)GAACUUCAGGGUCAGCUUGCCG | (ATTO590)GAmAmCUUCAmGGGUCAmGCUUGCCG |

**Table 4-14: List of used TLR stimuli**

| stimulus   | stock concentration | used concentration | source                          |
|--|---------------------|--------------------|---------------------------------|
| CpG-ODN 1668   | 250 µM              | 1 µM               | InvivoGen, Toulouse, France     |
| CpG-ODN 2216   | 250 µM              | 1 µM               | InvivoGen, Toulouse, France     |
| R848 (resiquimod)  | 1 mg/ml             | 1 µg/ml            | InvivoGen, Toulouse, France     |
| Pam <sub>3</sub> CSK <sub>4</sub>                                      | 1 mg/ml             | 1 µg/ml            | InvivoGen, Toulouse, France     |
| Lipopolysaccharid (LPS) from <i>Salmonella Minnesota</i> (smooth form) | 100 µg/ml           | 0.1 µg/ml          | provided by U. Seydel (Borstel) |

**Table 4-15: transfection reagents used in the study**

| Transfection reagent | Company                      |
|----------------------|------------------------------|
| Dotap                | Carl Roth, Karlsruhe         |
| Lipofectamine 2000   | life technologies, Darmstadt |

### 4.1.7 Kits

In Table 4-16 all kits used during the study are listed.

**Table 4-16: List of all used kits**

|   |                                      |
|---|--------------------------------------|
| BD OptEIA™ ELISA Set (IL12p40, IL6, TNF, IL1β)    | BD Bioscience Pharmingen, Heidelberg |
| CD14 MicroBeads human                             | Miltenyi Biotech, Bergisch Gladbach  |
| eBioscience ELISA (hIFN-α)                        | eBioscience, Frankfurt               |
| FastSYBR® Green PCR Master mix                    | Applied Biosystems, Darmstadt        |
| High-capacity cDNA transcription kit              | Applied Biosystems, Darmstadt        |
| Immobilon™ Western Chemilumineszenz HRP Substrate | Millipore, Schwalbach                |
| RNeasy® Mini Kit                                  | Qiagen, Venlo, Netherlands           |

## 4.2 Methods

### 4.2.1 Cell biology

#### 4.2.1.1 Cell culture of mammalian cells

All cells were grown at 37 °C, 5% CO<sub>2</sub> and 95% humidity in an incubator. Cell number of cell suspensions were determined by diluting cell suspension 1:10 in trypan blue solution staining exclusively dead cells. Cell numbers were count with Neubauer cell counting chamber.

#### 4.2.1.2 Generation of murine primary bone marrow-derived dendritic cells

Bone marrow cells were prepared from 4- to 10-week old mice. Therefore the mice were sacrificed by CO<sub>2</sub> asphyxia. Bone marrow was flushed out of tibiae and femurs with supplemented RPMI using a 27G syringe. The cells were singularized with a 20G syringe and count.

For differentiation of dendritic cells 8 x 10<sup>6</sup> cells were seeded in a 145 cm<sup>2</sup> tissue plate in 25 ml differentiation medium plus Granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF was derived from X-63 cells (supplied by M.Lutz, University Würzburg). They are cytoplastoma cells secreting murine GM-CSF. The amount was batch dependent. After 5 days the cells were fed by adding 25 ml differentiation medium and GM-CSF. At day 7 the non-

adherent, immature dendritic cells were harvested by centrifugation (1300 rpm, 5 min). Afterwards the cells were count and seeded for experiments.

#### **4.2.1.3 Isolation of human peripheral blood mononuclear cells**

For isolation of human peripheral blood mononuclear cells (PBMCs) blood from healthy donors were taken with a syringe containing heparin. The heparized blood was mixed with 1 x PBS in a ratio of 1:1 to 1:2 and added on top of Pancoll (d = 1,077 g/l) to separate the different compounds of the blood. After centrifugation (1800 rpm, 20 min, 4 °C, without brakes) the white ring between pancoll and serum was transferred in a new tube and filled up with PBS. The cells were washed by centrifugation (1300 rpm, 10 min, 4 °C) and resuspended in PBS. After a further centrifugation (1000 rpm, 15 min, 4 °C) to remove granulocytes, the cells were all put together and washed a last time with PBS. After centrifugation (1300 rpm, 10 min, 4 °C) the cells were resuspended in RPMI medium supplemented with 10% FCS and count with a Neubauer cell counting chamber.

#### **4.2.1.4 Isolation of CD14<sup>+</sup> cells**

CD14 positive monocytes were isolated by immunomagnetic cell separation which is based on magnetic antibody labeling (MACS-Microbeads). The labelled cells were separated by enrichment in a magnetic field and then eluted in a semi-automated manner from the column via AutoMACS device.

To isolate CD14 positive monocytes, fresh isolated PBMCs were used with CD14 microbeads according manufacturer's instructions. Thereby the CD14 positive cells were labeled and separated from the negative cells via AutoMACS Separator. The positive cells were eluted in the positive fraction by means of the program "possel". This represents the enriched CD14<sup>+</sup> monocytes. The cells eluted in the negative fraction were depleted of CD14<sup>+</sup> cells. The cells were centrifuged (1300 rpm, 10 min, 4 °C), resuspend in RPMI supplemented with 10% FCS and used as indicated in stimulation.

#### **4.2.1.5 Cell stimulation**

Human primary freshly isolated cells were transfected reverse. Therefore the cells were added on top of the transfection mixture. For ELISA and RNA analysis, PBMCs and CD14<sup>-</sup> cells were adjust to  $2 \times 10^6$  / ml, CD14<sup>+</sup> cells were adjust to  $1 \times 10^6$  / ml. For one duplicate, the transfection mixture was composed of 50 µl medium, stimulus and (3 µl/500 ng RNA) DOTAP (in case of RNA as stimulus). In case of inhibitory experiments, 500 ng/ml bRNA

and 1 µg/ml inhibitory or control RNA were used, corresponding to 108 nM. The mixture was incubated for 15 min at RT, before 170 µl medium were added. 100 µl were transferred in one well of a 96-well plate and 200 µl cells were added. The cells were incubated overnight if not indicated else in the experiment.

For western blot analysis, 50 µl transfection mixture was incubated for 15 min at RT and transferred in a 24-well plate.  $1 \times 10^6$  CD14<sup>+</sup> cells were added in a total volume of 300 µl.

The murine bone marrow derived dendritic cells were seeded in the wells minimal 2 h before transfection. The RNA was encapsulated in Lipofectamin (1 µl / µg RNA) for stimulation and incubated for 20 min at RT before adding to the cells. For ELISA 200 µl cell suspension ( $1 \times 10^6$  / ml) was seeded in a 96-well plate. The cells were incubated overnight.

#### 4.2.2 Confocal laser scanning microscopy

For fluorescence microscopy the confocal laser scanning microscope Leica TCS SP5 controlled by the Leica Application Suite Advanced Fluorescence (LAS AF) software was used. With the Argon ( $\lambda=488$  nm) and DPSS 561 ( $\lambda=561$  nm) laser the RNA labelled ATTO488 and ATTO590 were excited, respectively. Detectors were set corresponding to the emission spectra of the respective fluorescent dye (Table 4-17).

To avoid cross-emission between different fluorescent channels, sequential image acquisition was used. Z-offset and photo multiplier were set manually to obtain specific signal without detection of unspecific signals in negative controls.

**Table 4-17: Properties of the fluorescent dyes used for microscopy**

| fluorescent dye | used laser | $\lambda$ excitation | $\lambda$ emission |
|-----------------|------------|----------------------|--------------------|
| ATTO488         | Argon      | 480 - 515 nm         | 500 - 600 nm       |
| ATTO590         | DPSS561    | 575 - 610 nm         | 500 - 700 nm       |

Live-cell imaging was performed in a specialised incubation chamber, adjusting temperature to 37 °C and control CO<sub>2</sub> for 5% atmosphere. This chamber consists of Incubator S with Heating Insert P (PeCon, Erbach) controlled by Tempcontrol 37-2 digital (Leica) and CTI-Controller 3200 (Leica).

BMDCs were seeded in µ-Slides 8 well and incubated overnight. The live-cell imaging was started 1 h before transfection, to warm up Slides. The labelled RNAs (50 pmol each) were

co-transfected with Lipofectamine2000. The imaging was started as soon as possible after transfection.

Within one experiment, multiple visual fields were analysed in parallel using the “Mark and Find” mode of the LAS AF software in combination with a motorised high precision stage. The marked positions were recorded every minute over a time of 6 h.

### **4.2.3 Immunoassays**

#### **4.2.3.1 Enzyme-linked immunosorbant assay**

To quantify the amount of secreted cytokines, a sandwich ELISA was performed as indicated in the respective protocol of the manufacturers. Cell-free supernatants were diluted in PBS supplemented with 10% FCS (v/v) (blocking buffer) for measuring TNF, IL12p40, IL6 and IL1 $\beta$ . For hIFN- $\alpha$  the supernatants were diluted in PBS supplemented with 0.5% BSA (w/v) and 0.05% Tween (v/v). All experiments were performed two or three times as indicated. The cytokines were detected by measuring the absorbance at 490 nm with 650 nm as reference in a photometer (SUNRISE Absorbance reader, Tecan, Salzburg, Austria). Cytokine concentrations were calculated using a standard dilution of the respective cytokines. In the end, concentrations were calculated with Magellan V 5.0 software (Tecan, Salzburg, Austria). Standard determination was performed according to the 4 parameter fit regression.

#### **4.2.3.2 Co-Immunoprecipitation**

To test the direct binding of RNA and TLR7, co-immunoprecipitations were performed. Therefore, HEK cells expressing TLR7-HA were transfected with RNA and beads were added to pull out one of the binding partners. The presence of the other was checked.

HEKs growing in a 75 cm<sup>2</sup> flask were resuspended in 5 ml OptiMEM and 1 ml was used per sample. The cells were transfected with 3  $\mu$ g RNA (2 times 3  $\mu$ g for co-transfection) for 1 h at RT. Afterwards the cells were centrifuged (7 min, 1300 rpm, 4 °C) and washed with 1 ml PBS. The cells were lysed with lysis buffer on ice for 10 min. The cell lysis was centrifuged again (7 min, 1300 rpm, 4 °C) to remove cell debris. In parallel, the agarose beads (10  $\mu$ l / sample) were blocked with PBS 10% FCS for 1 h rotating at 4 °C. Afterwards, the beads were centrifuged (200 x g, 2 min, 4 °C) and washed with wash buffer. The beads were resuspended in 100  $\mu$ l / sample wash buffer and added to the cell lysis. The mixture was incubated for 3 h

rotating at 4 °C. The samples were centrifuged (200 x g, 2 min, 4 °C) and washed three times with wash buffer.

For analysing the amount of TLR7, meaning pulling out biotinylated RNA with streptavidin beads, 25 µl 2 x SDS was added to each sample and cooked 5 min at 95 °C. The samples were stored at -20 °C and used for western blot analysis (6% PAA-Gel) to detect the HA-TLR7.

For analysing the amount of RNA pulled down with TLR7-HA using anti-HA beads, 1 ml Trizol was added to each sample. The samples were stored at -20 °C for short storage and used for RNA isolation followed by cDNA synthesis and qPCR analysis for 16SrRNA.

## **4.2.4 Molecular biology**

### **4.2.4.1 RNA preparation from stimulated cells**

Total RNA was isolated from human PBMCs using peqGOLD Total RNA kit following manufacturer's protocol. RNA isolation included DNase digestion. The isolated RNA was stored at -80 °C or directly used for cDNA preparation.

### **4.2.4.2 cDNA synthesis**

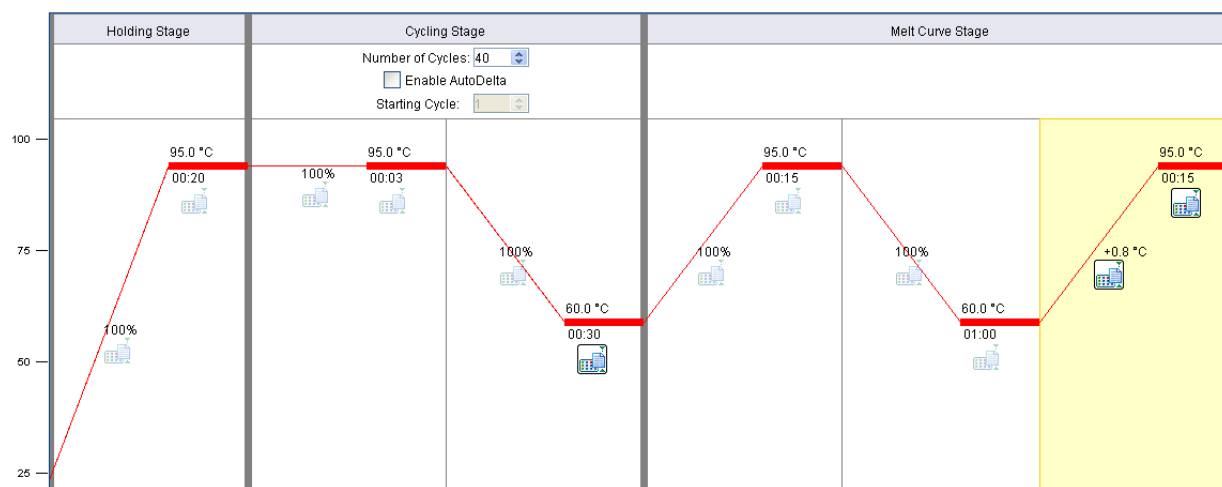
cDNA synthesis was performed with High-Capacity cDNA transcription kit. Thereby the messenger RNA was transcribed to DNA using random primers. 10 µl of RNA were mixed with 10 µl mastermix including buffer, primers, RNase inhibitors, dNTPs, Reverse Transcriptase. The mixture was transcribed according to the manufacturer's protocol. Afterwards, the cDNA (20 µl) was diluted with 40 µl H<sub>2</sub>O.

### **4.2.4.3 Quantitative PCR**

For quantification of cDNA the cyanid fluorescent dye SybrGreen was used. It interferes with double stranded DNA and enables to monitor amplicon development. For the setup 2 µl cDNA was mixed with 18 µl maser mix, including fast SybrGreen mastermix, water and primers for the observing gene. The gene expression was measured in duplicates and was performed in a 96 well plate. The measurement was operated by StepOne Plus Real-Time PCR System. Thereby the program for amplification was performed as shown in Figure 4-1. Afterwards the dissociation curves for each amplicon were analysed to determine the melting point. Automatic detection of baseline and threshold values was used. To calculate the relative expression the determined C<sub>t</sub> values were substrated from the C<sub>t</sub> value of a reference gene, which is constitutively expressed (β-actin), resulting in a ΔC<sub>t</sub> for each target gene.



The relative expression (rE) was then calculated as  $rE = 1/(2^{\Delta C_t})$ . As specificity controls no-template and no RT (cDNA synthesis without adding reverse transcriptase) samples were included.



**Fig. 4-1: Running program for qRT-PCR**

#### 4.2.4.4 Preparation of bacterial RNA

For preparation of bacterial RNA (bRNA) the indicated bacteria were grown over night in media at 37 °C shaking. Only Bifidobacteria were grown under anaerobic conditions. For gram-negative bacteria and *Staphylococcus aureus* LB medium were used. For the other gram-positive bacteria BHI media were used.

The culture was centrifuged for 10 min at 4000 x g and 4 °C. The supernatants were discarded and the pellets resuspend in 750 µl lysozyme (40 mg/ml) per 50 ml bacteria culture for 20 min at 37 °C to lyse the bacteria. Afterwards the samples were frozen at -80 °C and then resuspended in 5ml Trizol. After incubation at room temperature for 5 min, 1 ml Chloroform was added, vortexed and incubated for 2 min. The mixture was centrifuged for 60 min at 4000 x g, 4 °C. Then the upper fraction was carefully transferred in a new tube, 1 ml per tube and mixed each with 830 µl Isopropanol. After incubation at room temperature for 10 min the tubes were centrifuged for 10 min at 12000 x g, 4 °C. After removing the supernatant, 1 ml 75% ethanol was added in each tube and incubated for 5 min at room temperature. To sediment the RNA again, centrifugation at 7500 x g were performed for 5 min at 4 °C. The pellets were dried and resuspended in DEPC-H<sub>2</sub>O. The concentration was measured by means of the NanoDrop® ND-100 UV VIS Spectrophotometer. Aromatic rings of the RNA absorb the UV light at  $\lambda=260$  nm.

To ensure a RNA preparation free of bacterial contaminants, the isolated RNA was additionally isolated using RNeasy Mini Kit from Qiagen. Therefore 100 µg RNA per column was isolated according to the manufacturer's protocol. The concentration was again measured and RNA was stored at -80 °C.

#### 4.2.4.5 Preparation of bacterial tRNA

For preparation of isolated transfer RNA (tRNA) the whole bRNA preparation was separated by a denatured polyacrylamide gel electrophoresis and bands at the expected size were isolated.

Therefore a 10% PAGE containing 0.8 M urea was prepared and run without samples for 30 min at 200 V. Meanwhile the bRNA was mixed with loading dye. The pockets of the PAGE were rinsed with running buffer before the samples were loaded. The PAGE ran at 200 V for 2 h. The PAGE was removed from the apparatus and put on a paper for thin layer chromatography and detected with UV light. Then the tRNA bands were cut out, shredded in small pieces and put with 0.3 M Na<sup>+</sup>-Acetate in a tube. After shaking with 550 rpm overnight at room temperature to elute the RNA out of the gel, the supernatant was added on a column with a 0.45 µm pores and centrifuged at 2500 x g for 1 min. The flow through was transferred in a new tube and 2.5 V 100% cold ethanol was added and stored overnight at -20 °C. To precipitate the RNA, the sample was centrifuged at 16000 x g for 90 min. Afterwards the pellet was dried and resuspended in RNase free H<sub>2</sub>O. The concentration was determined by NanoDrop<sup>®</sup>.

#### 4.2.4.6 Preparation of bacterial 16S rRNA

To isolate 16S rRNA the whole RNA preparation of *Staphylococcus aureus* was used. The RNA was mixed with 2 x RNA loading dye and heated for 10 min at 70 °C before cooling down on ice for 3 min. The samples were put on a 1.5% low-melting agarose gel prepared with DEPC/TBE supplemented with SERVA Stain Clear G. The gel ran at 100 V. The bands were visualized by UV-light and the corresponding band cut out. After melting the agarose at 70 °C for 10 min, it was cooled down to 42 °C for 5 min. 1 U agarase was added per 100 mg agarose gel to digest the agarose and incubated at 42 °C for 30 min. Afterwards NH<sub>4</sub>-OAc was added to a final concentration of 2.5 M, incubated on ice for 3 min and centrifuged 10 min at 10000 rpm to separate the rest of the agarose gel from the RNA solution. The supernatant was transferred in a new tube and mixed well with 2/3 volume of phenol/chloroform. After incubation on ice for 10 min the mixture was centrifuged for 10 min at 13000 rpm, 4 °C. The upper aqueous phase was transferred in a new tube and mixed with 1 volume of chloroform. After centrifugation (5 min, 13000 rpm, RT) the upper phase was

supplemented with 0.1 volume of 5 M NH<sub>4</sub>-oAc and the RNA was precipitated with 2 volumes of 100% ethanol at -80 °C for 30 min. During centrifugation (30 min, 13000 rpm, 4 °C) the RNA was pelleted and dried before re-suspended in DEPC-H<sub>2</sub>O at 37 °C for 5 min. The RNA solution was measured by NanoDrop<sup>®</sup>.

#### 4.2.4.7 Ligation of different modivariants of tRNA<sup>Tyr</sup>

The ligation of the modivariants was performed by Steffen Kaiser (AG Helm), one of our cooperation partners in Mainz.

Splinted ligation was performed by annealing three synthetic fragments of RNA (IBA Göttingen; 5' fragment: GGUGGGGUUCCCGAGCGGCCAAAG; middle fragment: GGAGCAGACUGUAAAUCUGCCGUCACAG; 3' fragment: ACUUCGAAGGUUCGAUC CUUCCCCACCACCA), corresponding in sequence to unmodified E. coli tRNA<sup>Tyr</sup> or its modivariants/mutants onto a reverse complementary oligodeoxynucleotide (IBA Göttingen; TGGTGGTGGGGGAAGGATTCGAACCTTCGAAGTCTGTGACGGCAGATTTACAGTC TGCTCCCTTTGGCCGCTCGGGAACCCACC). Appropriate fragments (4 nmol) were 5'-phosphorylated by incubating in KL buffer (50 mM Tris-HCl [pH 7.4], 10 mM MgCl<sub>2</sub>) supplemented with 5 mM ATP, 5 mM DTT, and 0.75 units/l T4 polynucleotide kinase (PNK, Fermentas) in a final volume of 150 µl in the thermomixer at 37 °C for 1 h. To the phosphorylation reaction mixture, an equimolar amount of the 5' fragment and the DNA splint were added, as well as KL buffer, ATP (5 mM), and DTT (5 mM), leading to a final volume of 500 µl and an 8 µM concentration of each fragment. The RNA fragments were hybridized to the DNA splint by heating to 75 °C in the thermomixer for 4 min and letting the reaction mixture cool down to room temperature for 15 min. Then, T4 DNA ligase (1.5 units/µl; Fermentas) and T4 RNA ligase 2 (22 ng/µl) were added, and the ligation was performed in the thermomixer at 16 °C overnight. Template DNA was removed by addition of 1.5 units/µl DNase I (Fermentas), followed by 1 h of incubation at 37 °C. The tRNAs were purified from ligation mixtures by denaturing PAGE, excised and eluted from the gel, and precipitated with ethanol. Concentrations were calculated from absorption at 254 nm, as determined on a Nanodrop ND-1000 spectrometer after resuspension in water. (Steffen Kaiser, University Mainz)

## 4.2.5 Biochemistry

### 4.2.5.1 Preparation of protein lysates

According to the experiments the cells were seeded in cell culture plates. For stimulation the cells were preincubated with medium containing less FCS before stimulated as mentioned in the experiments. After indicated timepoints, the plates were centrifuged (1 min, 13000 rpm) to attach the cells to bottom and the medium was carefully removed. The cells were washed with cold 1 x PBS. The cells were lysed in 100 – 150  $\mu$ l freshly prepared RIPA lysis buffer supplemented with protease and phosphatase inhibitors and SDS for 10 min on ice. Afterwards the cell suspension was transferred in a tube and cooked for 5 min at 95 °C. The lysates were stored at -20 °C.

### 4.2.5.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins due to their molecular weight. Thereby SDS eliminates non-covalent bindings and charges the proteins negatively, whereby the amount of negative charge correlates with the length of the protein. Therefore the proteins can be separated by gel electrophoresis moving towards the positive electrode depending on their molecular weight.

Up to 50  $\mu$ l of each samples were loaded on a polyacrylamide gel, the percentage was dependent on the proteins of interest and the gel was run at constant voltage for 1.5 to 2 hours. A prestained protein marker was used as internal reference for size. Proteins from the gel were further analyzed by western blotting.

### 4.2.5.3 Western blot analysis

Proteins separated by SDS-PAGE were blotted on a nitrocellulose membrane (Millipore, Billerica, USA) by semidry blotting procedure with 1.57 mA/cm<sup>2</sup> membrane for 90 min using blocking buffer. To block unspecific binding sites, the membrane was incubated with blocking buffer for 1 h at RT on a vertical shaker. Afterwards, proteins were detected using a specific primary antibody diluted as indicated in blocking buffer and incubated over night at 4 °C on a vertical shaker. Membranes were washed three times with washing buffer before adding horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:4000 in blocking buffer for 1 h at room temperature. After three further wash steps with washing buffer bound antibodies were detected by chemiluminescence using enhanced luminol. Light emission was detected with a cooled CCD camera device.

#### **4.2.6 Statistical analysis**

All experiments were performed at least two times and the number of experiments is stated in the legends. Means + SD are shown. Significant differences were evaluated by Student's ttest in Microsoft Excel. Data were analyzed by means of GraphPad prism 4.03 program (GraphPad Software, San Diego California, USA). The stimulation curves for calculation of IC<sub>50</sub> were calculated by R using the four-parameter Weibull functions<sup>195,196</sup>. p<0.05 (\*), p<0.01 (\*\*), p<0.001 (\*\*\*) were considered significant.

## 5 RESULTS

This work was an analysis of the role of the naturally occurring 2'O-methylation modification Gm18 in *E.coli* tRNA<sup>Tyr</sup> and its effect on innate immunity. Specifically, 2'O-methylation-mediated inhibition of immune activation was studied in the work for this thesis. Underlying mechanisms and structural properties responsible for immunoinhibition by 2'O-methylated RNA were analysed.

### 5.1 Inhibition by 2'O-methylated RNA

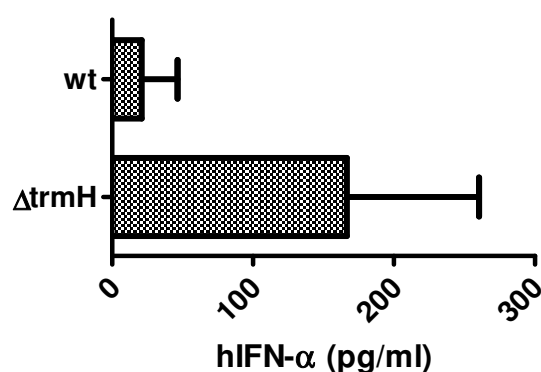
#### 5.1.1 Inhibition of IFN- $\alpha$ secretion by *E. coli* tRNA<sup>Tyr</sup>

Access to pure native RNA species is limited. Thus, previous studies either used *in vitro* transcripts or short synthetic oligoribonucleotides to analyse the effects of specific RNA modifications on the immunostimulatory capacity. Moreover, most studies have been based on observations of self RNA and have not addressed the role of naturally occurring modifications in non-self RNAs. For example, 2'O-methylation has been shown to have suppressive effects on IFN- $\alpha$  secretion from pDCs via TLR7 activation when incorporated into synthetic RNAs, whereas its role in foreign RNA has not been analysed and discussed. This is of special interest because the modification also occurs in bacterial RNA<sup>179,185,197,198</sup>. Therefore, in cooperation with AG Helm from the University of Mainz, we analysed the relevance of modifications in bacterial RNAs. Several native tRNAs and their corresponding *in vitro* transcripts (which therefore lacked the naturally occurring modifications) were tested for immunostimulation. It was found that native *E. coli* tRNA<sup>Tyr</sup> was not stimulatory while the corresponding transcript induced IFN- $\alpha$ <sup>123</sup>. The difference in immunostimulation had to be due to RNA modifications, as this was the only difference between the native form and the transcript. Further experiments identified guanosine 2'O-methylation at position 18 (Gm18) as the modification responsible for inhibition of immunostimulation of plasmacytoid DCs<sup>123</sup>.

##### 5.1.1.1 2'O-methylation in natural bacterial tRNAs

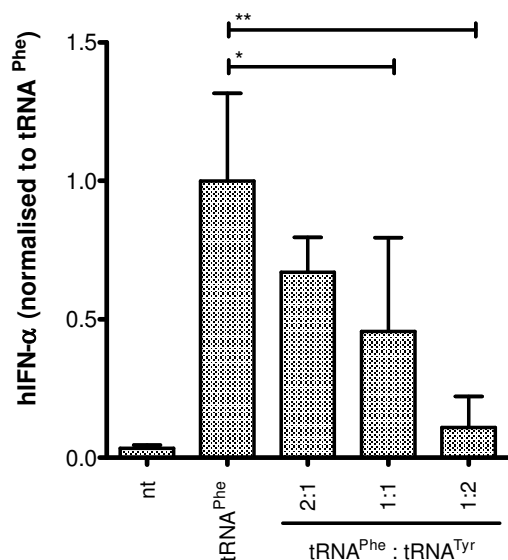
In *E. coli*, the protein TrmH is responsible for the 2'O-methylation of guanosine at position 18 (Gm18) in tRNA. It is the gene product of *trmH* and acts as a 2'-O-methyltransferase<sup>185</sup>. Thirteen out of 45 tRNAs in *E. coli* carry a methylation at position G18, and *trmH* deficiency results in loss of this methylation<sup>198</sup>. To determine the influence of 2'O-methylated tRNAs on

the stimulation efficiency of bacterial RNA preparations, total tRNA preparations from wild-type (wt) and a TrmH mutant *E. coli* ( $\Delta trmH$ ) were isolated and used to transfect PBMCs. Induction of IFN- $\alpha$  is shown in Figure 5-1. While the wt tRNA induced only marginal amounts of IFN- $\alpha$ , the tRNA preparation from the TrmH mutant enhanced the production of IFN- $\alpha$ . This indicated an important effect of the 2'-O-methylation at position G18 on immune stimulation. Surprisingly, stimulation with wt tRNA was very inefficient, even though 32 of 45 tRNAs showed no methylation at position 18 and should therefore be stimulatory. The results suggested that the methylation modification mediated more than just immunosilencing.



**Fig. 5-1: Methyltransferase TrmH influences the stimulatory potential of *E. coli* tRNA.** Human PBMCs were stimulated overnight with a whole tRNA preparation (500 ng/ml) derived from wild-type *E. coli* (wt) or a *trmH* deletion mutant of *E. coli* ( $\Delta trmH$ ). IFN- $\alpha$  production was analysed by ELISA using cell-free supernatants. The data are mean values (+ SD) of two independent experiments.

We hypothesised that the 2'-O-methylation not only prevents stimulatory activity but is also capable of inhibiting stimulation by unmodified tRNAs (by acting in a dominant inhibitory manner). To test this hypothesis, the native Gm18-containing *E. coli* tRNA<sup>Tyr</sup> was used in co-transfections in different ratios with native, stimulatory tRNA<sup>Phe</sup> lacking Gm18. tRNA<sup>Phe</sup> was previously shown to induce IFN- $\alpha$  in pDCs<sup>123</sup>. The IFN- $\alpha$  production in this experiment is shown in Figure 5-2. At tRNA<sup>Phe</sup>:tRNA<sup>Tyr</sup> ratios of 1:1 and 1:2, stimulation was significantly reduced. The results suggested that tRNA<sup>Tyr</sup> is not only non-stimulatory but also has immunoinhibitory effects on TLR7-mediated IFN- $\alpha$  production. For a detailed analysis of the inhibition, a neutral non-stimulatory - which was also uninhibitory - was included.



**Fig. 5-2: *E. coli* tRNA<sup>Tyr</sup> is capable of inhibiting simulation by tRNA<sup>Phe</sup>.** *E. coli* tRNA<sup>Phe</sup> (500 ng/ml) with or without *E. coli* tRNA<sup>Tyr</sup> was used to transfect human PBMCs. The concentration of *E. coli* tRNA<sup>Phe</sup> was kept constant but it was combined with *E. coli* tRNA<sup>Tyr</sup> in the ratios indicated. The mixtures were encapsulated in DOTAP. The DOTAP volume was adjusted for the total amount of RNA. IFN- $\alpha$  levels were determined from cell-free supernatants by ELISA. The data represent the mean values (+ SD) from three independent donors. The data are normalised to the amount of IFN- $\alpha$  induced by tRNA<sup>Phe</sup> alone, to reduce donor-related variations. nt, not treated (negative control); \* $p < 0.05$ ; \*\* $p < 0.01$

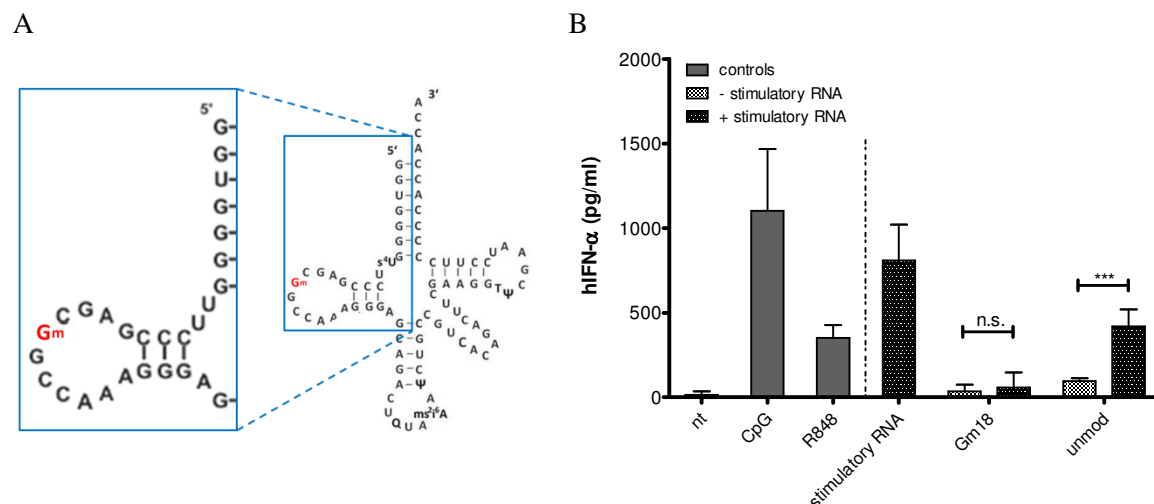
### 5.1.1.2 Small portions of *E. coli* tRNA<sup>Tyr</sup> are sufficient to inhibit immunostimulation when incorporating 2'O-methylation

Individual, fully modified native tRNAs are difficult to isolate. Likewise, preparation of a whole tRNA transcript bearing a single Gm18 modification is very time consuming<sup>199</sup>. We therefore wanted to establish a tool to study the mechanisms of inhibition more easily. We wanted to determine whether a small fragment of a given tRNA containing the inhibitory Gm18 modification would be sufficient to inhibit stimulatory RNA.

To test the hypothesis, we ordered a commercially synthesised oligoribonucleotide of 27 nucleotides, of the same sequence as nucleotides 1–27 of tRNA<sup>Tyr</sup> and with incorporation of a 2'O-methylation at position 18 (Gm18). The oligoribonucleotide was tested for immunostimulation and for its ability to inhibit the stimulation of co-transfected RNA in a dominant manner. As control, the same fragment without any modification (“unmod”) was used (Figure 5-3). Both oligoribonucleotides (ORNs) failed to induce IFN- $\alpha$  when administered to PBMCs alone (grey bars). This was unexpected for the control oligoribonucleotide, yet it might indicate that a length exceeding 27 bps was necessary or that certain sequence motifs should be present to induce immunostimulation. After co-transfection with stimulatory bacterial RNA, IFN- $\alpha$  production was reduced by more than 90% with the methylated RNA oligonucleotide, while IFN- $\alpha$  secretion with the unmodified RNA oligonucleotide was reduced by less than 50% (black bars). We therefore concluded that the tRNA fragment with the methylation at position 18 was sufficient for abrogation of immunostimulation. The small



Gm18 was used for all future experiments and it was called inhibitory RNA. The unmodified version of the RNA fragment was used as the control.



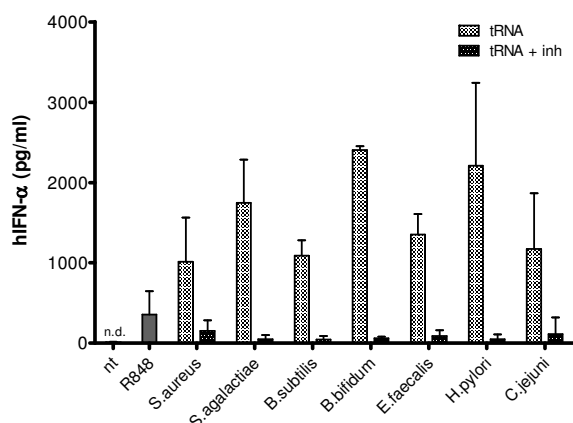
**Fig. 5-3: A short fragment of the *E. coli* tRNA<sup>Tyr</sup> with 2'O-methylation at position G18 was found to be sufficient for the immunosilencing effects.** (A) The structure of natural tRNA<sup>Tyr</sup> is shown on the right and the smaller fragment used for stimulation experiments is shown on the left. (B) Human PBMCs were stimulated with short oligoribonucleotides (as in A; 1 µg/ml) with (Gm18) or without (unmod) 2'O-methylation at position 18, and co-transfected overnight with (black bar) or without (grey bar) stimulatory RNA (500 ng/ml). Cell-free supernatants were assessed for IFN-α by ELISA. CpG-DNA (1 µM) and R848 (1 µg/ml) were used as positive controls to stimulate pDCs; nt, untreated negative control; The data are mean values (+ SD) of 3 pooled independent experiments, each done in duplicates.

### 5.1.1.3 Immunostimulation of tRNA and total RNA prepared from different bacterial species

Next, we wanted to know whether other RNA preparations from bacterial species other than *E. coli* would fail to induce IFN-α. This would help to identify other bacteria that use modifications as potential immune escape mechanisms and to find other immunosilencing or immunoinhibitory modifications. Also, we wanted to determine whether stimulatory tRNA preparations from different bacterial species could be inhibited by our inhibitory RNA preparation.

tRNA preparations from a range of bacterial species were therefore isolated, including gram-positive and gram-negative bacteria, pathogenic bacteria, and commensal bacteria. Human PBMCs were transfected with the tRNA preparations - alone or together with inhibitory RNA - and the production of IFN-α was measured. As shown in Figure 5-4, all the tRNA preparations tested induced release of IFN-α in concentrations ranging from 1 ng/ml to 2.5 ng/ml. Thus, no other immunosilencing tRNA preparations were identified.

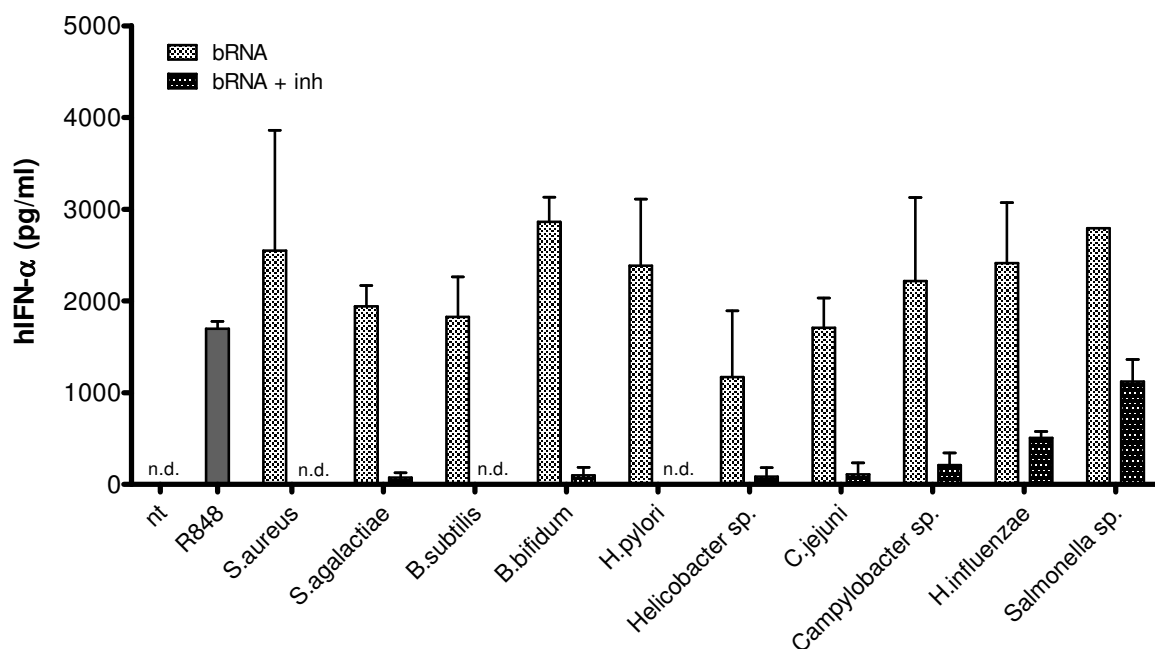
After adding inhibitory RNA to the tRNA preparations, the amount of IFN- $\alpha$  secreted decreased for all bacterial species (Figure 5-4), indicating that the immunoinhibitory effect influenced the stimulation of tRNAs of a diverse set of bacterial species.



**Fig. 5-4: tRNAs from different bacterial species induced IFN- $\alpha$  and could be inhibited by our inhibitory RNA.** Whole tRNA preparations (500 ng/ml) of *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus subtilis*, *Bifidobacterium bifidum*, *Enterococcus faecalis*, *Helicobacter pylori* and *Campylobacter jejuni* were used alone (grey bars) or together with 1  $\mu$ g/ml inhibitory RNA (inh; black bars) to transfect human PBMCs overnight. Cell-free supernatants were used to measure the production of IFN- $\alpha$ . R848 (1  $\mu$ g/ml) was used as positive control. nt, untreated negative control; n.d., not detectable. The data are mean values (+ SD) of 3 independent experiments.

It was found that the inhibitory RNA was able to suppress stimulation by a single tRNA and by total tRNA preparations from different bacterial species. In a preparation of total RNA, in addition to tRNA, mRNA, rRNA, and different small RNAs are present. To test whether these RNAs were also stimulatory and whether stimulation by total RNA extracts could also be inhibited, total RNA (bRNA) preparations from various bacterial strains were isolated and tested. The preparations were tested for their stimulatory capacity and for their ability to be inhibited by the methylated inhibitory RNA. All bRNA preparations were capable of inducing IFN- $\alpha$  production ranging from 1 ng/ml to 3 ng/ml (Figure 5-5). The concentration of RNA was the same as for tRNA preparations, and the stimulatory effects were also in the same range. Stimulation induced by bRNA preparations was reduced by over 90% after co-transfection with inhibitory RNA, except for bRNA of *Salmonella sp* and *H. influenzae* (Figure 5-5). Here, the stimulation decreased by only 50% and 70%, respectively.

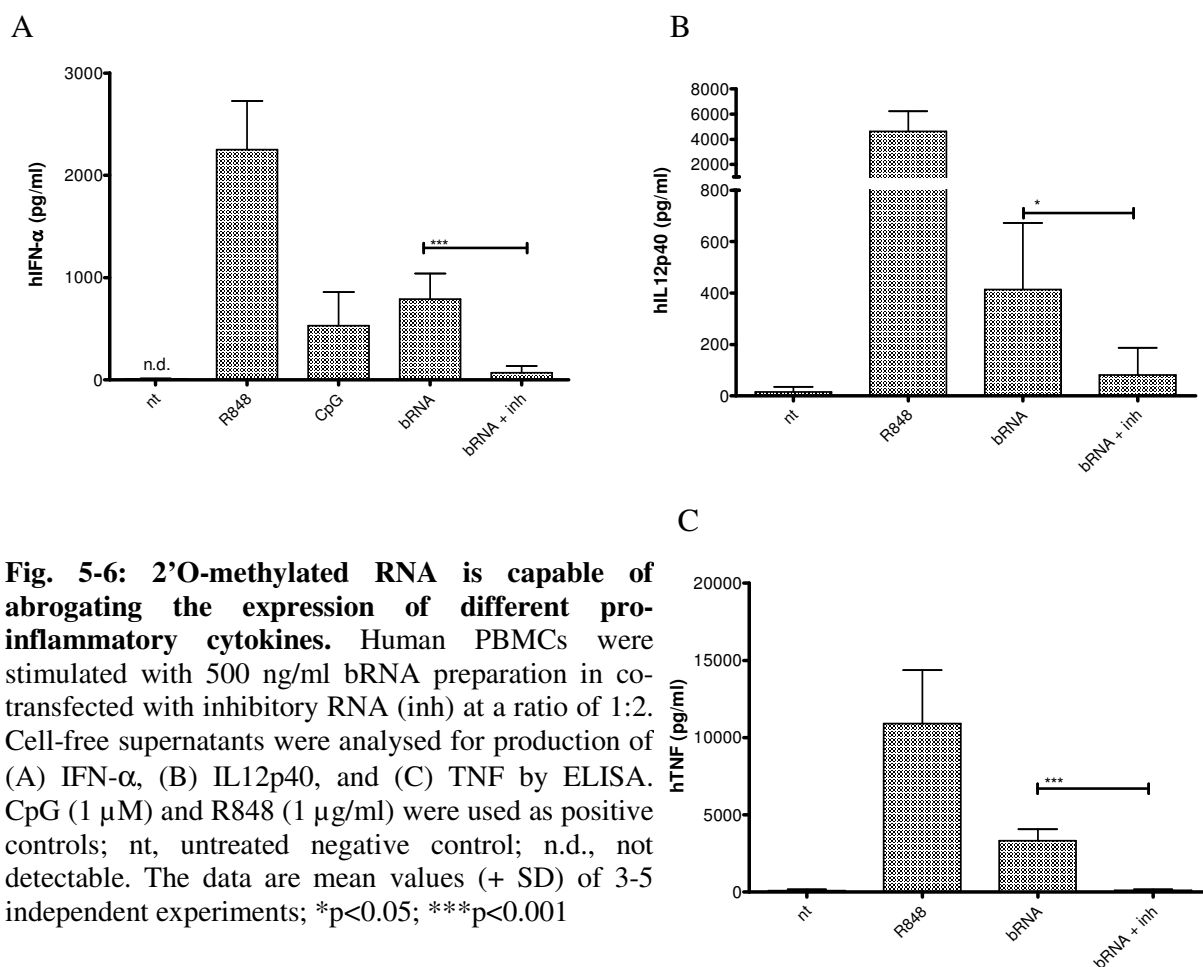
Unless otherwise indicated, in all the experiments that follow the total RNA preparation of *S. aureus* was used as total bRNA.



**Fig. 5-5: Total RNA preparations from different bacterial species were able to induce IFN- $\alpha$  and stimulation could be inhibited by inhibitory RNA.** Whole RNA preparations (500 ng/ml) from different bacteria were used to transfect human PBMCs overnight, alone (grey bar) or together with (black bar) 1  $\mu$ g/ml inhibitory RNA (inh). Production of IFN- $\alpha$  was measured by ELISA. CpG (1  $\mu$ M) and R848 (1  $\mu$ g/ml) were used as positive controls. nt, untreated negative control; n.d., not detectable. The data are mean values (+ SD) of 3 independent experiments, each done in duplicate.

### 5.1.2 Inhibition of other pro-inflammatory cytokines

Most of the data reported in the literature concerning 2'-O-methylation of RNA and suppression of immunostimulation were derived from human PBMCs that were tested for IFN- $\alpha$  production. IFN- $\alpha$  in PBMCs is specific for pDC stimulation via TLR7<sup>123,187,190</sup>. Other pro-inflammatory cytokines produced after activation of other cell types have not yet been investigated in detail. To determine the influence of other cell types in PBMC preparations, we measured the production of IL12p40 and TNF. PBMCs were stimulated with bRNA co-transfected with inhibitory RNA. As shown in Figure 5-6, bRNA was able to induce the secretion of pro-inflammatory cytokines IL12p40 and TNF in addition to IFN- $\alpha$ . Co-transfection with bRNA and inhibitory RNA led to a significant decrease in the production of IL12p40 and TNF. TNF and IL12p40 are not the characteristic cytokines produced by pDCs; these cells express TLR7 and TLR9 and they are known to mainly produce IFN- $\alpha$  and IL6<sup>200</sup>. Therefore, we concluded that bRNA is also capable of activating receptors other than TLR7, as TLR7 is exclusively expressed on pDCs and B cells. This other receptor has to recognise bRNA and is inhibited by inhibitory RNA.

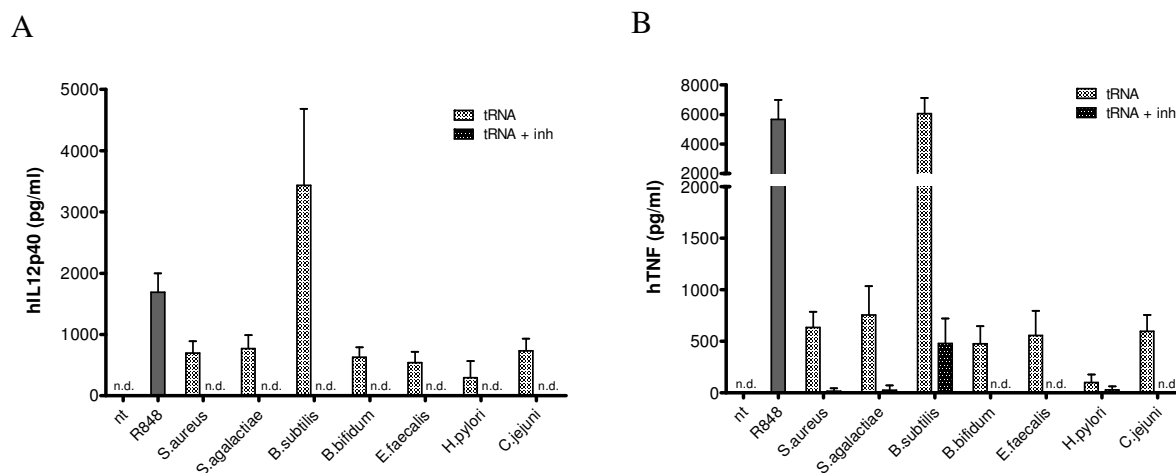


**Fig. 5-6: 2'O-methylated RNA is capable of abrogating the expression of different pro-inflammatory cytokines.** Human PBMCs were stimulated with 500 ng/ml bRNA preparation in co-transfected with inhibitory RNA (inh) at a ratio of 1:2. Cell-free supernatants were analysed for production of (A) IFN- $\alpha$ , (B) IL12p40, and (C) TNF by ELISA. CpG (1  $\mu$ M) and R848 (1  $\mu$ g/ml) were used as positive controls; nt, untreated negative control; n.d., not detectable. The data are mean values (+ SD) of 3-5 independent experiments; \* $p$ <0.05; \*\*\* $p$ <0.001

It was observed above that tRNA and bRNA preparations from various bacterial species induce IFN- $\alpha$  production and that for all RNAs, IFN- $\alpha$  production could be inhibited by adding our inhibitory RNA. Next, we tested whether these particular RNA preparations could also induce pro-inflammatory cytokine production and whether this again could be inhibited.

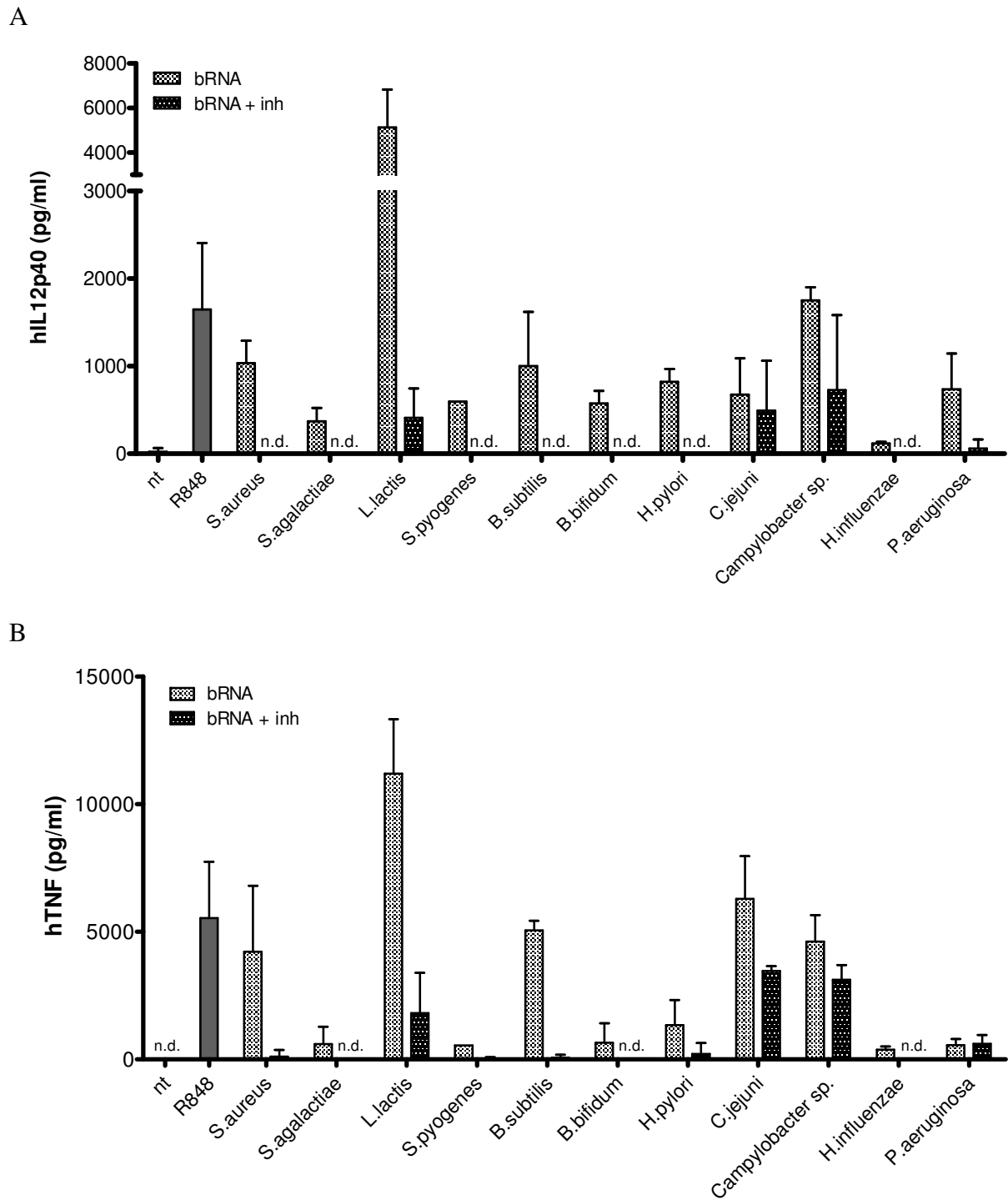
As shown in Figure 5-7, all the tRNA preparations tested led to production of IL12p40 and TNF (grey bars). Most tRNAs led to a cytokine production of between 500 and 1000 pg/ml - except for *B. subtilis*, which induced up to 3.5 ng/ml IL12p40 and 6 ng/ml TNF.

Co-transfection with inhibitory RNA at a ratio of 1:2 greatly reduced the production of the cytokines induced by tRNA preparations from all the bacterial species tested (black bars).



**Fig. 5-7: tRNA preparations from different bacterial species were found to be capable of inducing pro-inflammatory cytokines, and the induction could be inhibited by inhibitory RNA.** tRNA preparations (500 ng/ml) from different bacteria were used to transfect human PBMCs overnight, alone (grey bars) or together with (black bars) 1  $\mu$ g/ml inhibitory RNA (inh). Production of (A) IL12p40, and (B) TNF was measured in cell-free supernatants by ELISA. R848 (1  $\mu$ g/ml) was used as positive control. nt, untreated negative control; n.d., not detectable. The data are mean values (+ SD) of 3 independent experiments, each done in duplicates.

As the tRNA preparations caused induction of pro-inflammatory cytokines and the induction could be inhibited by inhibitory RNA, total bRNA preparations from different bacterial species were also tested. As illustrated in Figure 5-8, all the bRNA preparations tested were able to induce the cytokines analysed, but to somewhat different levels. While most of the bRNA preparations induced IL12p40 production to levels of 1 ng/ml or less, the bRNA of *L. lactis* induced production of up to 5 ng/ml IL12p40. For TNF, most of the bRNA preparations induced production of the cytokine at levels less than 5 ng/ml; only *L. lactis* induced production of around 12 ng/ml TNF. For all the bRNA preparations, they had been prepared by the same method and the same amount of RNA was used for transfection. On co-transfection with inhibitory RNA, the induction by most bRNAs decreased by at least 90%. For the two *Campylobacter* species tested, no relevant decrease in secretion of IL12p40 and TNF could be measured. This may have been due to contaminations with LPS.



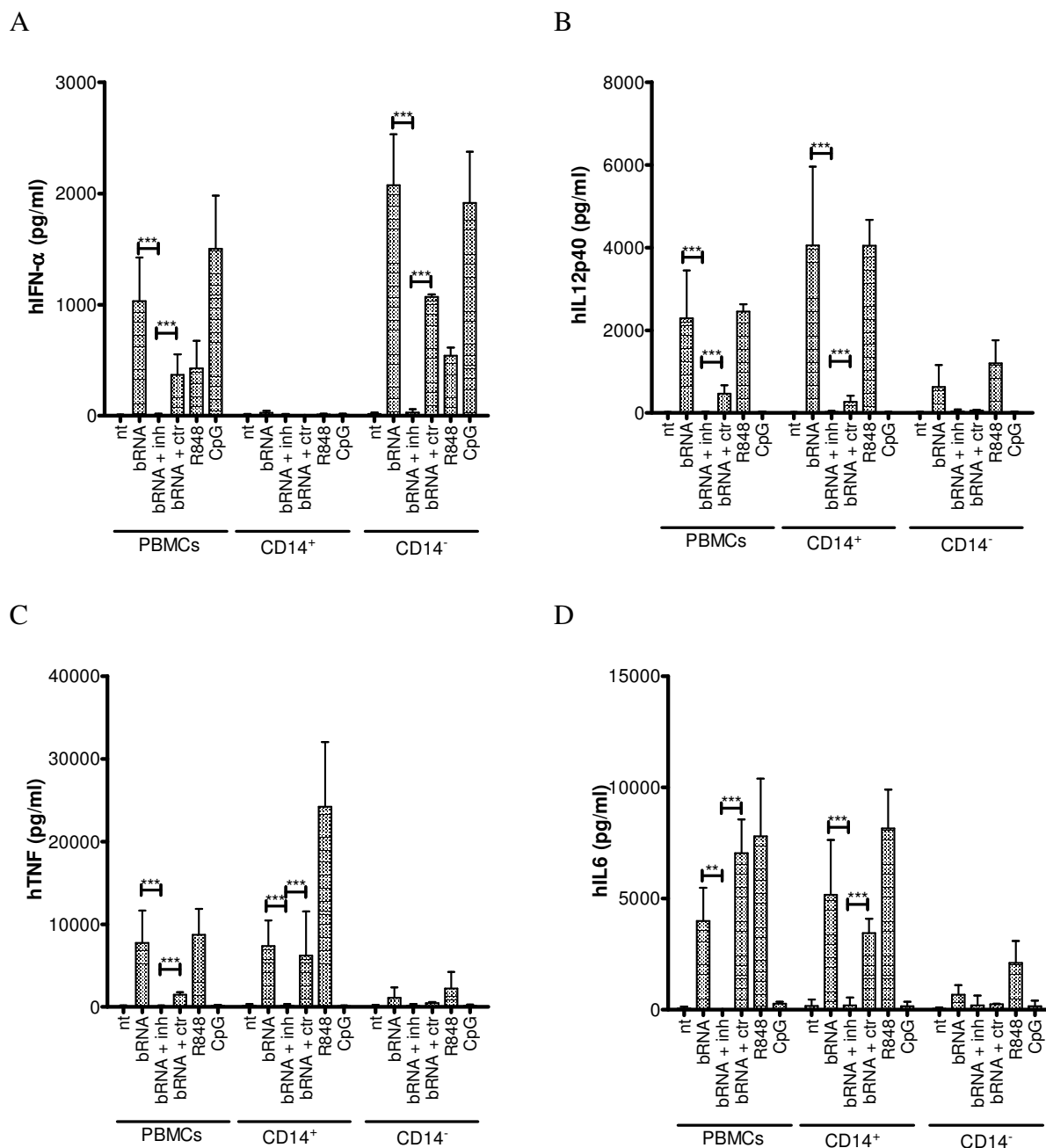
**Fig. 5-8: Total bRNA preparations from different bacterial species were found to be capable of inducing production of pro-inflammatory cytokines, and the induction could be inhibited by inhibitory RNA.** Total bRNA preparations (500 ng/ml) of different bacteria were used to transfect human PBMCs overnight, alone (grey bars) or together with (black bars) 1  $\mu$ g/ml inhibitory RNA (inh). Production of (A) IL12p40, and (B) TNF was measured in cell-free supernatants by ELISA. R848 (1  $\mu$ g/ml) was used as positive control. nt, untreated negative control; n.d., not detectable; The data are mean values (+ SD) of 3 independent experiments.

### 5.1.2.1 Pro-inflammatory cytokines and IFN- $\alpha$ are secreted from different cell types

In the previous section, we observed the induction of pro-inflammatory cytokines in response to bRNA. Because TLR7 is a nucleic acid-sensing receptor that recognises bRNA that is restricted to induction of type-I interferons and expression in pDCs, other cell types and receptors must recognise bRNA.

In the literature, it has been observed that different nucleic acid receptors are expressed in different cell types: TLR7 and TLR9 are expressed mainly in pDCs and less in B cells, while TLR8 is mainly expressed in monocytes and only weakly in NK cells and T cells <sup>102</sup>. We therefore investigated the relevance of monocytes for production of certain pro-inflammatory cytokines in response to bRNA, as both IL12p40 and TNF are cytokines known to be secreted from monocytes.

Monocytes were isolated by selection for CD14 expression and transfected with bRNA. In Figure 5-9, the cytokine expression was compared to that in CD14-negative cells and whole PBMCs. IFN- $\alpha$  production was found in PBMCs and CD14-negative cells, while secretion of TNF, IL12p40, and IL6 was found in PBMCs and monocytes. For all cell types and all tested cytokines, the stimulation by bRNA alone was inhibited by co-transfection with inhibitory RNA, but it was not reduced when bRNA was used in co-transfections with control RNA (Figure 5-9). For IL12p40, the cytokine induction by bRNA co-transfected with control RNA was not as high as for bRNA alone, but it was significantly higher than after transfection with bRNA along with inhibitory RNA. This observation might be due to competition between bRNA and control RNA for receptor binding, but the effect was not dominant, as the addition of inhibitory RNA reduced the signal even more. As monocytes are known to express high levels of TLR8, but not TLR7, it is possible that TLR8 in monocytes is responsible for production of pro-inflammatory cytokines in response to bRNA. Furthermore, cytokine induction could be inhibited by inhibitory RNA, raising the possibility that TLR8 can also be inhibited by 2'O-methylated RNA.



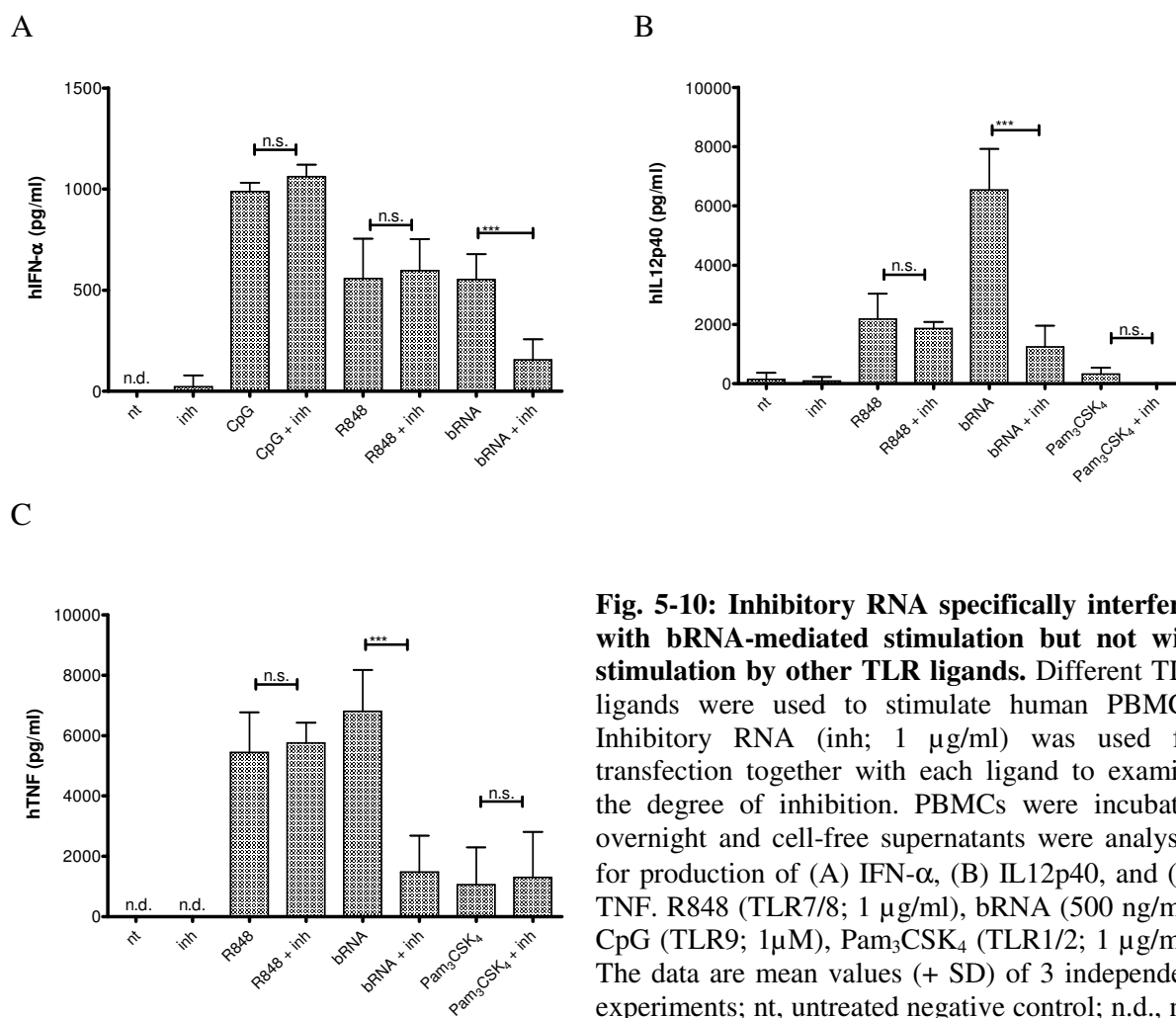
**Fig. 5-9: Pro-inflammatory cytokines are expressed by monocytes in response to stimulation by bRNA.** Human PBMCs were used to isolate CD14-positive monocytes by MACS separation. PBMCs ( $2 \times 10^6$ /ml) and CD14-positive ( $1 \times 10^6$ /ml) and CD14-negative fractions ( $2 \times 10^6$ /ml) were used for stimulation. bRNA (500  $\mu$ g/ml) was used for co-transfection overnight with inhibitory or control RNA (each 1  $\mu$ g/ml). Production of (A) IFN- $\alpha$ , (B) IL12p40, (C) TNF, and (D) IL6 was measured by ELISA. CpG (1  $\mu$ M) and R848 (1  $\mu$ g/ml) were used as positive controls; CD14-positive fraction: > 90% CD14-positive, CD14-negative fraction: < 2% CD14-positive nt, untreated negative control; n.d., not detectable; The data are mean values (+ SD) of 3–6 independent experiments.



### 5.1.2.2 Inhibitory RNA does not inhibit immunostimulation by other TLR ligands

As shown previously, inhibitory RNA was able to abrogate immunostimulation by different bacterial RNA species. This was observed in different cell types and for different cytokines. In the next step, we wanted to determine whether inhibition was specific for RNA recognition or whether other TLRs could also be inhibited, indicating an unspecific effect. Thus, different TLR ligands were used to stimulate PBMCs together with transfection by inhibitory RNA. CpG-DNA, R848, and Pam<sub>3</sub>CSK<sub>4</sub> were used as ligands for TLR9, TLR7/8, and TLR1/2, respectively<sup>30,201,202</sup>. R848 was selected because it activates the same receptor as bRNA. CpG-DNA activates TLR9, another nucleic acid-sensing TLR that is located in endosomes and specifically expressed in pDCs and B cells. Pam<sub>3</sub>CSK<sub>4</sub> activates the dimer TLR1/2, a receptor for components of the cell wall of gram-positive bacteria that is located on the cell surface. In preliminary tests, the TLR ligands were titrated to find concentrations outside of the saturation range in order to make any inhibition apparent. As CpG-DNA activates TLR9, which is mainly expressed in pDCs, this stimulation was only tested for induction of IFN- $\alpha$ . Furthermore, Pam<sub>3</sub>CSK<sub>4</sub> is not sensed by pDCs as they do not express any TLR1/2 dimer, resulting in no induction of IFN- $\alpha$ . Thus, this set-up was only analysed for secretion of IL12p40 and TNF.

As shown in Figure 5-10, all TLR ligands were able to induce cytokines; only Pam<sub>3</sub>CSK<sub>4</sub> failed to induce production of significant amounts of IL12p40 in PBMCs. After addition of inhibitory RNA, the amounts of cytokines induced showed no significant differences - even for R848. Only the induction by bRNA, as positive control, was reduced after addition of inhibitory RNA. As the cytokine production induced by bRNA is suppressed, but not that induced by R848, this finding indicates that there may be different recognition mechanisms for the two TLR7 ligands.

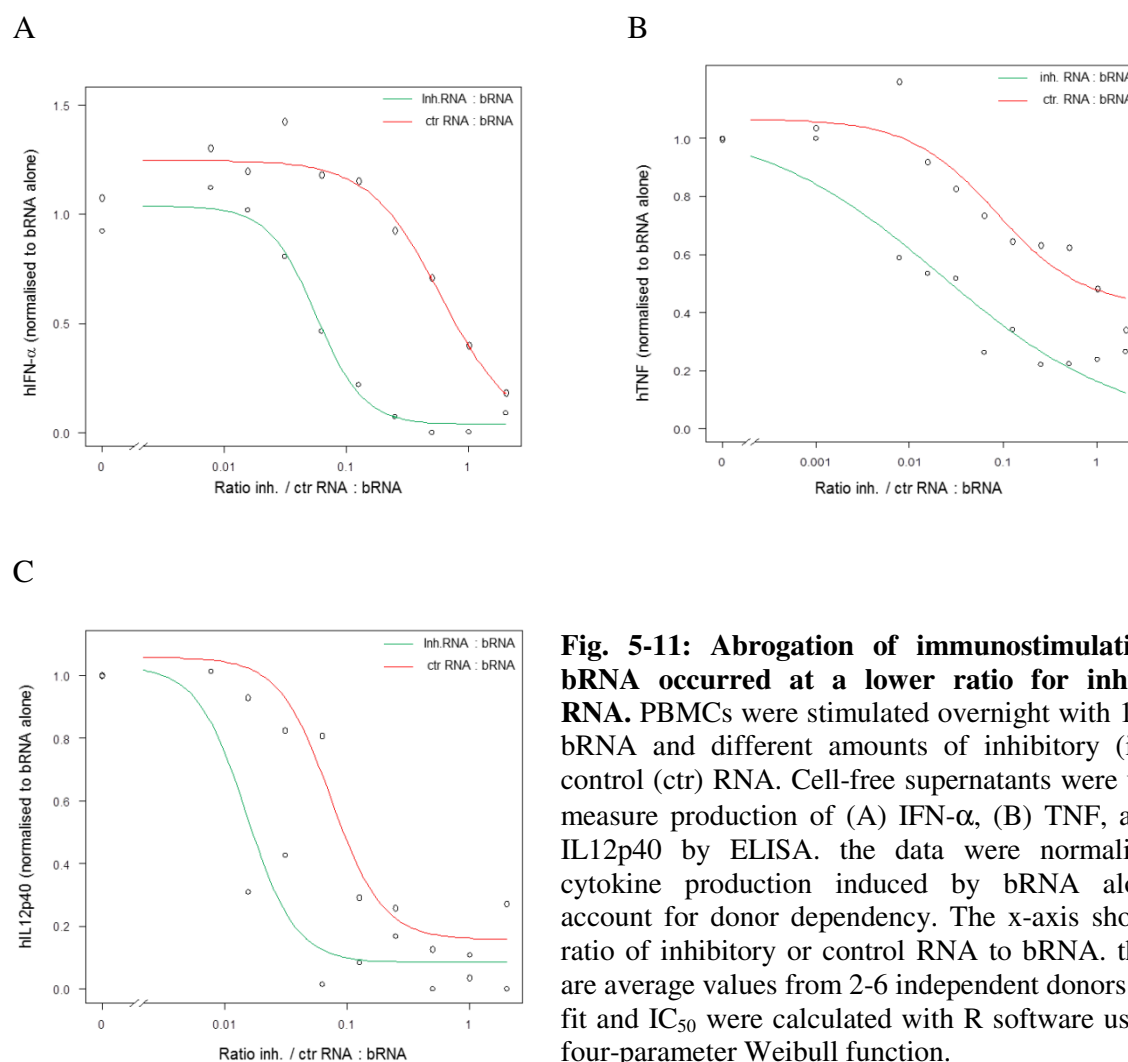


**Fig. 5-10: Inhibitory RNA specifically interferes with bRNA-mediated stimulation but not with stimulation by other TLR ligands.** Different TLR ligands were used to stimulate human PBMCs. Inhibitory RNA (inh; 1  $\mu$ g/ml) was used for transfection together with each ligand to examine the degree of inhibition. PBMCs were incubated overnight and cell-free supernatants were analysed for production of (A) IFN- $\alpha$ , (B) IL12p40, and (C) TNF. R848 (TLR7/8; 1  $\mu$ g/ml), bRNA (500 ng/ml), CpG (TLR9; 1 $\mu$ M), Pam<sub>3</sub>CSK<sub>4</sub> (TLR1/2; 1  $\mu$ g/ml). The data are mean values (+ SD) of 3 independent experiments; nt, untreated negative control; n.d., not detectable; n.s., not significant; \*\*\* p<0.001.

### 5.1.2.3 2'-O-methylated RNA suppress immunostimulation by bRNA more efficient than unmodified, nonstimulatory RNA

In the previous sections, we have seen that inhibitory RNA can reduce the production of different cytokines induced by bacterial RNA. In the next step, we wanted to know whether the inhibitory RNA had the same efficiency for all cytokines and receptors. The inhibitory RNA was therefore titrated to a fixed concentration of bRNA to determine the concentration necessary for 50% inhibition (IC<sub>50</sub>). Titration curves with control RNA were performed in parallel as controls. The titration curves are shown in Figure 5-11. For all cytokines, secretion decreased after addition of inhibitory or control RNA. Yet, the inhibition was much more efficient when inhibitory RNA was added. The IC<sub>50</sub> values were calculated for all curves using four-parameter Weibull function, and are shown in Table 5-1. The IC<sub>50</sub> has been normalised to the cells and not to the receptor, as the experiments were performed *in vitro* and not with isolated receptor. *In vitro*, the binding of the RNA and the receptor is influenced by

different cellular properties such as uptake and transport, as well as cytokine production. Thus, the binding of the RNA to the receptor might result in different  $IC_{50}$  values for analysis with isolated receptors. The  $IC_{50}$  of the inhibitory RNA differed for the various cytokines. It was higher for IFN- $\alpha$  than for TNF. This might indicate a lower efficiency in inhibiting the IFN- $\alpha$  response. The  $IC_{50}$  for the control RNA was about 10 times higher for IFN- $\alpha$  and TNF than that for the inhibitory RNA. Yet, for TNF the curve fit was not optimal, as the curve for bRNA with control RNA did not fall to zero, showing that there was incomplete inhibition.



**Fig. 5-11: Abrogation of immunostimulation by bRNA occurred at a lower ratio for inhibitory RNA.** PBMCs were stimulated overnight with 1  $\mu$ g/ml bRNA and different amounts of inhibitory (inh) or control (ctr) RNA. Cell-free supernatants were used to measure production of (A) IFN- $\alpha$ , (B) TNF, and (C) IL12p40 by ELISA. the data were normalised to cytokine production induced by bRNA alone to account for donor dependency. The x-axis shows the ratio of inhibitory or control RNA to bRNA. the data are average values from 2-6 independent donors. Curve fit and  $IC_{50}$  were calculated with R software using the four-parameter Weibull function.

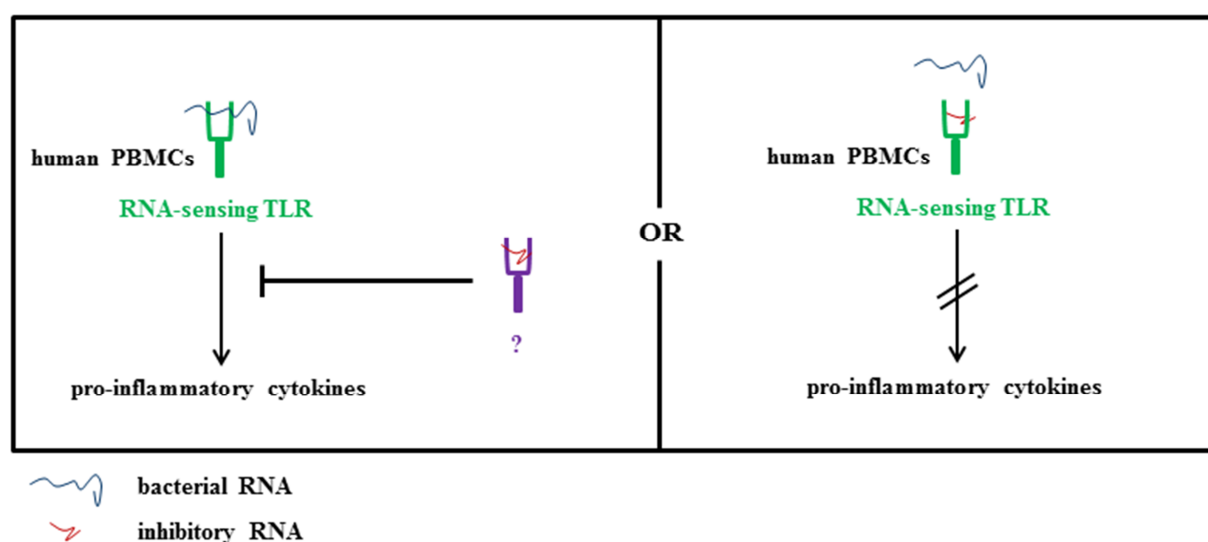
**Table 5-1: IC<sub>50</sub> of the inhibitory RNA was less than the IC<sub>50</sub> of control RNA.**

| cytokine                        | IC <sub>50</sub> of inhibitory RNA<br>(ratio bRNA : inhibitory RNA) | IC <sub>50</sub> of control RNA<br>(ratio bRNA : control RNA) |
|---------------------------------|---|---|
| <b>hIFN-<math>\alpha</math></b> | 0.060 $\mu$ g/ml  | 0.637 $\mu$ g/ml  |
| <b>hTNF</b>                     | 0.009 $\mu$ g/ml  | 0.119 $\mu$ g/ml  |
| <b>hIL12p40</b>                 | 0.019 $\mu$ g/ml  | 0.084 $\mu$ g/ml  |

### 5.1.3 Mechanisms of inhibition by 2'O-methylated RNA

As inhibitory 2'O-methylated RNA was able to abrogate the activation of various cytokines by different RNA species, it is possible that different mechanisms are involved in this inhibitory effect. On the one hand, the inhibitory RNA could act as a competitive antagonist by binding to the same receptor. On the other hand, a further receptor could be present, sensing inhibitory RNA and then initiating an inhibitory pathway to repress positive signals of the RNA-sensing TLRs. The two hypotheses are depicted in Figure 5-12. To test which hypothesis might be correct, I investigated the repression of activation on different levels of the signalling pathway and analysed the binding of inhibitory RNA to TLR7.

To determine which hypothesis was most correct, we investigated the repression of activation at different levels of the signalling pathway and analysed the binding of inhibitory RNA to TLR7.

**Fig. 5-12: Models for the mechanism of inhibition.**

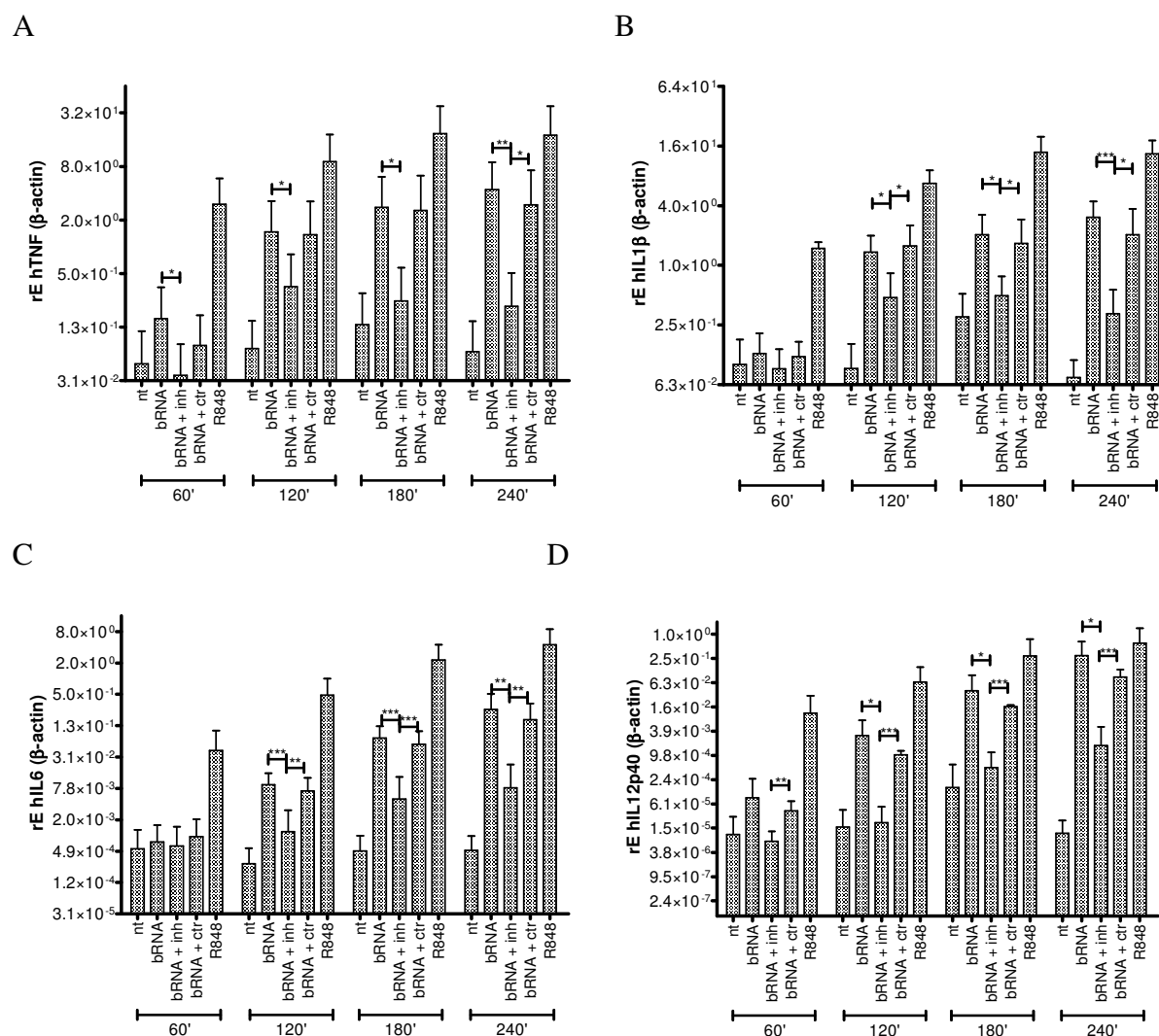
### **5.1.3.1 Inhibition of TLR activation by modified 2'O-methylated RNA is a rapid event**

Up to now, the repressive effect had always been observed after overnight incubation. To determine the level at which inhibition occurs, earlier time points and upstream effector events were examined.

If the inhibitory RNA binds directly to the same receptor as the stimulatory RNA, inhibition should be observed at all time points. If, on the other hand, inhibitory RNA binds to another receptor, leading to a blocking of the RNA signal, an induction of the signalling might be observed at early time points. To investigate this, human PBMCs were co-transfected with bRNA and inhibitory or control RNA. At different time points (60 min, 120 min, 180 min, and 240 min), the cells were lysed and the expression of mRNA encoding different cytokines was analysed by qRT-PCR (Figure 5-13). Investigation of the mRNA levels was done because ELISA would not detect cytokine release at these early time points.

For all the cytokines investigated, induction by R848 stimulation could be detected, starting early - after 1 h. The induction increased further at later time points. For stimulation with bRNA, only slight induction was found after 1 h. For later time points, the cytokine induction increased further. For co-transfection with the inhibitory RNA, no or only slight induction could be measured for all time points. Co-transfection with bRNA and control RNA induced similar levels of cytokines as bRNA alone. For all cytokines, the expression after stimulation with R848 as positive control started earlier and reached higher levels (Figure 5-13). This could be because bRNA consisted of complex molecules and was packaged in liposomes, while R848 was a small molecule that did not need any liposomes to be taken up into the cell. Thus, the recognition for R848 might be faster than for bRNA. For all cytokines, the expression was significantly higher after stimulation with bRNA alone than after co-transfection with inhibitory RNA. For all cytokines, co-transfection with bRNA and control RNA led to similar expression levels as transfection with bRNA alone.

Together, these data showed that inhibition by 2'O-methylated RNA occurred at early time points, indicating that a direct blocking of the signal might have taken place.



**Fig. 5-13: Expression of cytokines decreased at early time-points after addition of inhibitory RNA.** Human PBMCs were transfected with 1  $\mu\text{g/ml}$  bRNA together with and without 2  $\mu\text{g/ml}$  inhibitory (inh) or control (ctr) RNA. The cells were lysed at the indicated time points, RNA was isolated, and cDNA was synthesised. qPCR was performed for  $\beta$ -actin, TNF, IL1 $\beta$ , IL6, and IL12p40. The expression values of the cytokines were normalised to that of  $\beta$ -actin. The data are mean (+ SD) of three independent donors. R848 (1  $\mu\text{g/ml}$ ) was used as positive control. nt, untreated negative control; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

### 5.1.3.2 Inhibitory RNA blocks activation of downstream signalling

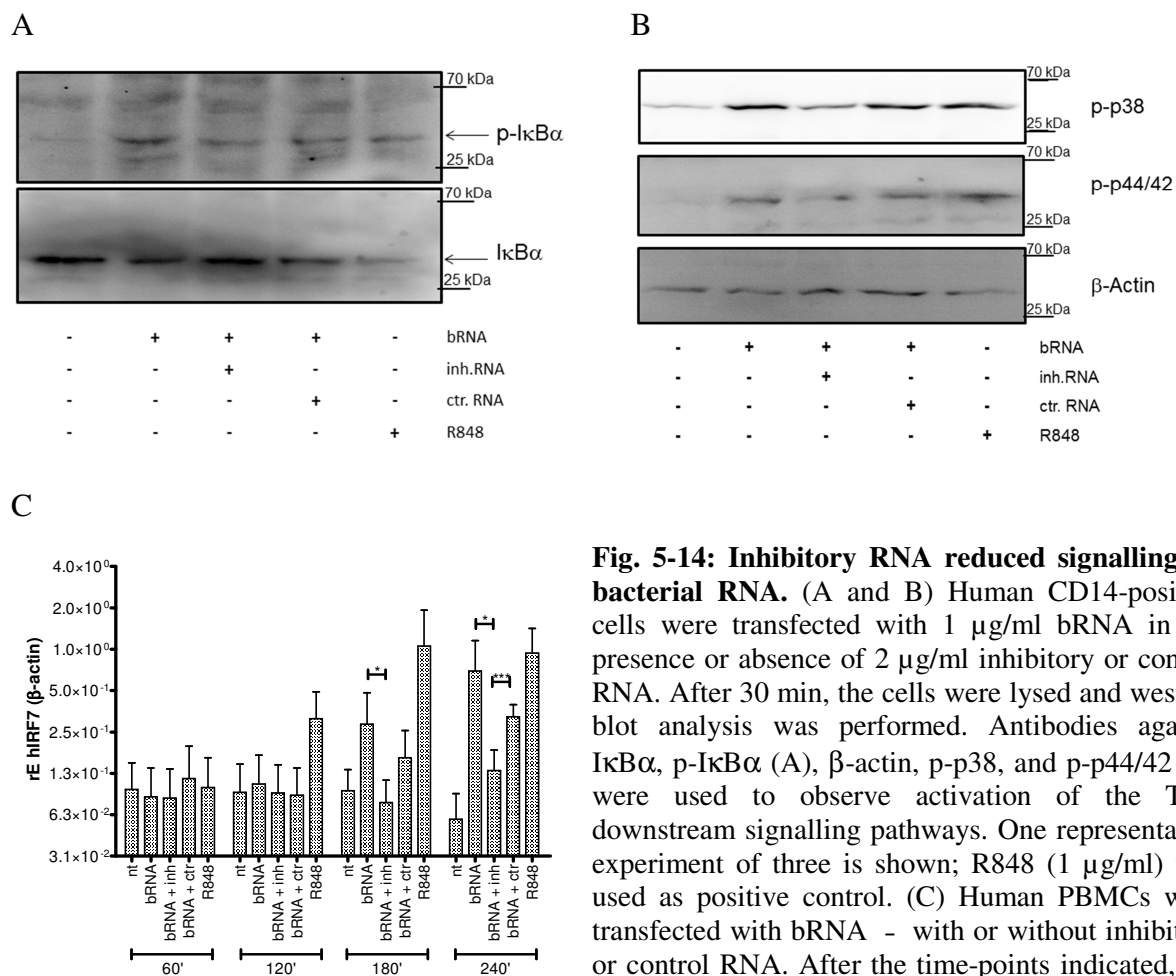
Cytokine expression (according to the level of mRNA) was inhibited by 2'-O-methylated RNA at all time points, indicating that the inhibition process started far upstream in the signalling cascade. Thus, in the next step I investigated whether the signalling cascade activated by bRNA was influenced when inhibitory RNA was added. I analysed the three main signalling pathways activated by TLR signalling, namely activation of MAPK, NF $\kappa$ B, and IRF. Activation of the MAPK pathway leads to phosphorylation of p42/44, p38, and JNK1/2. Activation of the NF $\kappa$ B pathway leads to phosphorylation and degradation of I $\kappa$ B- $\alpha$ , resulting in activation

of NF $\kappa$ B. Both pathways induce the expression of pro-inflammatory cytokines, i.e. of TNF and IL12p40. The third route that can be activated by TLR signalling leads to phosphorylation and enhanced expression of IRFs. Which IRF is activated will depend on the activated receptor. Activation of TLR7 leads to induction of IRF7, and TLR3 activation leads to IRF3 induction. The IRF pathway results in a strong type-I interferon response.

To study the effect of the inhibitory RNA on the different pathways, phosphorylation of different signalling intermediates was examined. Monocytes (CD14-positive cells) were therefore isolated from PBMCs and stimulated for 30 min. Monocytes were used as they are responsible for the production of pro-inflammatory cytokines, including TNF and IL12p40, in our set-up. By using one defined cell type, the background produced by unstimulated cells could be reduced. In Figure 5-14A, it can be seen that I $\kappa$ B- $\alpha$  was phosphorylated after stimulation with bRNA alone, bRNA plus control RNA, and after stimulation with R848. Only slight phosphorylation was observed after co-transfection with both bRNA and inhibitory RNA. Likewise, I $\kappa$ B- $\alpha$  was degraded upon transfection with bRNA alone, bRNA plus control RNA, and upon stimulation with R848, but not after stimulation with bRNA plus inhibitory RNA - indicating that inhibitory RNA blocked the activation of the NF $\kappa$ B pathway by bRNA.

Similar results were obtained for the MAPK pathway (Figure 5-14B). For p38 and p42/44, phosphorylation was observed after stimulation with bRNA alone, bRNA with control RNA, or R848. The untreated control and the cells co-transfected with bRNA plus inhibitory RNA showed no or only slight phosphorylation of MAPKs. Together, these data indicated that both pathways, the MAPK and the NF $\kappa$ B pathway, were inhibited by the inhibitory RNA.

To study the influence of the inhibitory RNA on the IRF pathway, the induction of IRF7 expression was measured by qRT-PCR analysis. IRF7 acts as transcription factor and is activated by phosphorylation downstream of TLR7 activation. Activation also leads to increased expression of IRF7. As shown in Figure 5-14C, expression of IRF7 increased after stimulation with R848 for 2 h and after transfection of RNA for 3 h, as observed for the expression of cytokines (Figure 5-13). After 3 h and 4 h, the induction of IRF7 by bRNA alone was significantly higher than induction by bRNA transfected together with inhibitory RNA. The co-transfection of bRNA together with control RNA was not significantly reduced. This result indicated that the IRF pathway was also inhibited.



**Fig. 5-14: Inhibitory RNA reduced signalling by bacterial RNA.** (A and B) Human CD14-positive cells were transfected with 1  $\mu\text{g/ml}$  bRNA in the presence or absence of 2  $\mu\text{g/ml}$  inhibitory or control RNA. After 30 min, the cells were lysed and western blot analysis was performed. Antibodies against I $\kappa$ B $\alpha$ , p-I $\kappa$ B $\alpha$  (A),  $\beta$ -actin, p-p38, and p-p44/42 (B) were used to observe activation of the TLR downstream signalling pathways. One representative experiment of three is shown; R848 (1  $\mu\text{g/ml}$ ) was used as positive control. (C) Human PBMCs were transfected with bRNA - with or without inhibitory or control RNA. After the time-points indicated, the cells were lysed, RNA was isolated, and cDNA was

synthesised. qPCR was performed for IRF7 and  $\beta$ -actin. The values of IRF7 were normalised to  $\beta$ -actin; R848 (1  $\mu\text{g/ml}$ ) was used as positive control. nt, untreated control; The data are mean values (+ SD) of 2 independent donors; \* $p < 0.05$ ; \*\*\* $p < 0.001$

### 5.1.3.3 Stimulatory and inhibitory RNA must be packaged together for inhibition

Up to this point, all experiments had been performed with RNA that had been introduced in co-transfections, i.e. the stimulatory RNA and the inhibitory RNA were mixed before being encapsulated with DOTAP for transfection and endosomal delivery. This procedure would have ensured uptake of both RNAs in the same cell and the same endosome. We wanted to determine whether this co-localisation was necessary and whether pre-stimulation of the cells with the stimulatory bRNA or blocking with inhibitory RNA would alter the efficiency of inhibition of cytokine production. Thus, both RNAs were encapsulated separately and used to transfect PBMCs at different time points. After incubation overnight, the release of cytokines was measured. The results are shown in Figure 5-15.

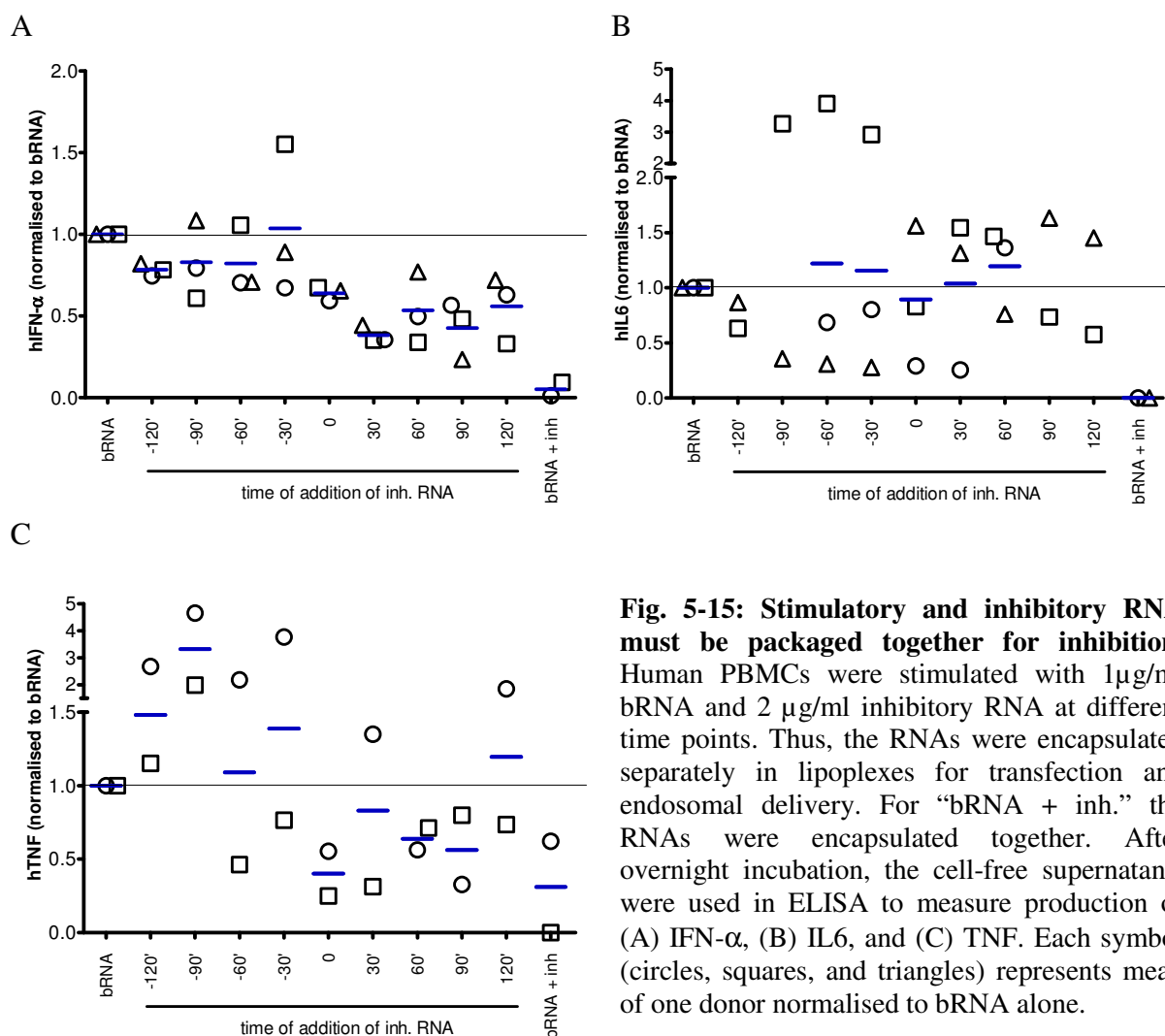


For IFN- $\alpha$  production (Figure 5-15A), a slight inhibition could be observed with all approaches. Interestingly, approaches in which the inhibitory RNA was added after the stimulatory RNA showed less IFN- $\alpha$  production (down to 20% of that for bRNA alone) than the approaches involving pre-treatment with the inhibitory RNA (down to 60% of that for bRNA alone). No approach was as efficient as co-transfection with both RNAs. Even transfection with stimulatory and inhibitory RNA at the same time, but individually encapsulated, reduced the stimulation down to 70% of that for bRNA alone.

For IL6 production (Figure 5-15B), the results were not as clear as for IFN- $\alpha$ . First, there were greater standard deviations. For one donor, the IL6 production was four times increased for pre-transfection with inhibitory RNA compared to transfection with bRNA alone. For the other donors, the cytokine production was decreased down to 20% of the stimulation by bRNA alone for pre-treatment with inhibitory RNA. For pre-stimulation with stimulatory bRNA, only one donor showed reduced IL6 production (down to 20%); the others had increased cytokine release compared to stimulatory RNA alone. Co-transfection reduced the IL6 production completely for all donors.

Ninety minutes of pre-treatment of the cells with inhibitory RNA resulted in five times more TNF than was induced by the bRNA alone (Figure 5-15C). Transfection with both separately encapsulated RNAs at the same time reduced the TNF production to 45% of bRNA that for bRNA alone. Addition of inhibitory RNA to bRNA resulted in a decrease in TNF production down to 50% of that for bRNA alone. Only pre-stimulation by bRNA for 2 h enhanced TNF production to 120% of that for bRNA alone. Again, co-transfection was the most efficient and least variable method of inhibition of cytokine production.

In summary, pre-treatment of the cells with inhibitory RNA reduced the amount of inhibition, and in some cases the cytokine production was even enhanced. The most efficient inhibition was observed after co-transfection with both RNAs. Yet, addition of inhibitory RNA up to 2 h after stimulation reduced the amount of cytokine secretion even when the bacterial RNA was delivered separately.

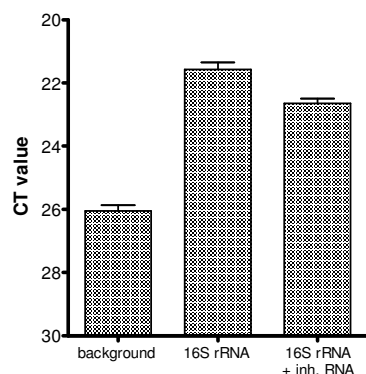


**Fig. 5-15: Stimulatory and inhibitory RNA must be packaged together for inhibition.** Human PBMCs were stimulated with 1 $\mu$ g/ml bRNA and 2  $\mu$ g/ml inhibitory RNA at different time points. Thus, the RNAs were encapsulated separately in lipoplexes for transfection and endosomal delivery. For “bRNA + inh.” the RNAs were encapsulated together. After overnight incubation, the cell-free supernatants were used in ELISA to measure production of (A) IFN- $\alpha$ , (B) IL6, and (C) TNF. Each symbol (circles, squares, and triangles) represents mean of one donor normalised to bRNA alone.

#### 5.1.4 Inhibitory RNA acts as an antagonist for TLR7 signalling

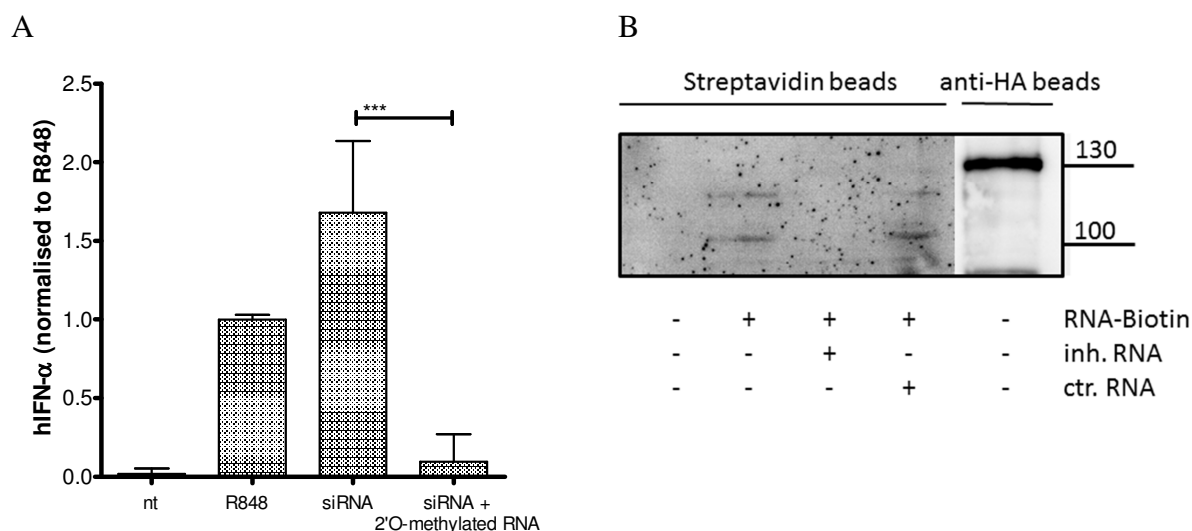
The above results indicated that modified RNA inhibits early events in TLR7 signalling. Thus, 2'-O-methylated RNA might bind directly to the receptor stimulated by bRNA, block it, and thereby prevent activation. To test this hypothesis, HEK293 cells stably expressing HA-tagged TLR7 were transfected with 16S rRNA of *S. aureus* in the absence or presence of inhibitory RNA. The cells were lysed and the RNA was pulled down by isolating the TLR7 protein with  $\alpha$ -HA beads. RNA bound to the protein was isolated with TRIzol and cDNA synthesis was performed. The cDNA was analysed by qPCR using 16S rDNA primers from a conserved region. The  $C_T$  values are plotted in Figure 5-16. They indicate the amount of 16S rRNA bound to the receptor, whereby a lower  $C_T$  indicates more RNA bound. The results suggested that 16S rRNA bound to TLR7. After addition of inhibitory RNA to the cells, the amount of 16S rDNA detected decreased by 1 cycle, indicating that the amount of 16S rRNA was reduced by 50% as the DNA is doubled in each cycle. This indicated that the

inhibitory RNA might have displaced the 16S rRNA from the receptor. 16S rDNA could also be detected in the background control. This might be due to contamination of the reagents used. Compared to the samples of bRNA plus inhibitory RNA, only one-sixteenth of the 16S rDNA was detected in the background control.



**Fig. 5-16: 16S rRNA may bind directly to TLR7 and can be displaced by 2'-O-methylated, inhibitory RNA.** HEK-TLR7HA cells were transfected with 3  $\mu\text{g/ml}$  16S rRNA from *S. aureus* with or without inhibitory RNA (3  $\mu\text{g/ml}$ ) for 1 h. The cells were lysed and anti-HA agarose beads were added for 3 h. Afterwards, the samples were washed and RNA was isolated with TRIzol. The 16S rRNA was detected with qRT-PCR using 16SrDNA primers from conserved regions. One representative experiment of three is shown.

To verify these first results, the experiment was switched around and the amount of receptor bound to RNA was analysed. Thus, HEK293 cells stably expressing HA-tagged TLR7 (131 kDa) were transfected with a short biotinylated and stimulatory siRNA. The siRNA was used because it was known to stimulate immune activation and could be bought with a biotin tag. The stimulatory capacity of the siRNA is shown in Figure 5-17A, where it was used to transfect PBMCs. The ability of 2'-O-methylated RNA to inhibit the stimulation by the siRNA is also shown. The biotinylated RNA was isolated with streptavidin beads and co-immunoprecipitation of TLR7 was analysed by western blot analysis using an HA antibody. The co-immunoprecipitation of TLR7-HA with biotinylated RNA is shown in Figure 5-17B. For HEK cells transfected with biotinylated RNA alone, two bands were detected. The same bands were observed for co-transfection with biotinylated RNA and control RNA, but not for co-transfection with inhibitory RNA. Both bands resulted from proteins smaller than the expected size of TLR7-HA (131 kDa). For TLR7-HA pulled down with anti-HA beads, one band of the expected size was detected. The proteins detected in the two bands after co-immunoprecipitation might be smaller due to cleavage of TLR7 during processing in the endosome or by activation (see Discussion). Yet, it was not formally proven whether the bands represented TLR7, and for this purpose an anti-TLR7 antibody should be used.

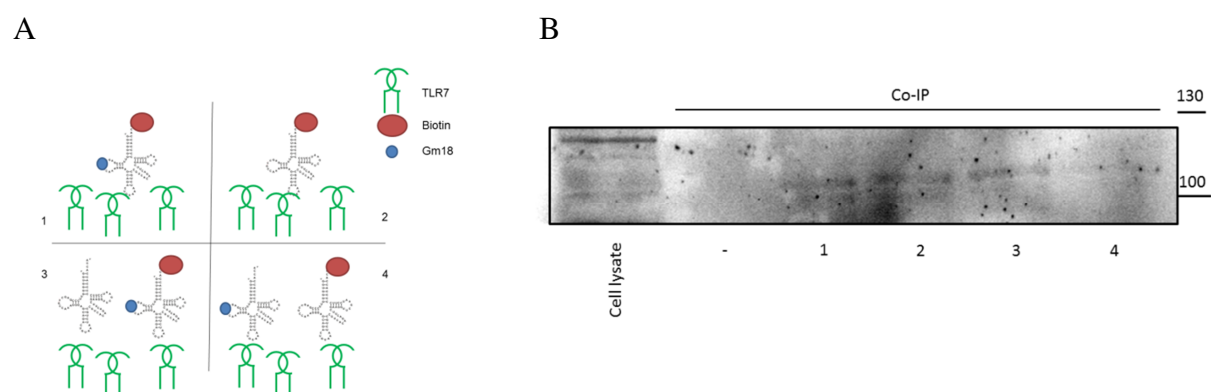


**Fig. 5-17: TLR7 is replaced from the stimulatory RNA by the 2'O-methylated RNA.** (A) 500  $\mu$ M siRNA was used alone or together with 500  $\mu$ M 2'O-methylated RNA to transfect PBMCs. After overnight incubation, cell-free supernatants were analysed for IFN- $\alpha$  content. R848 (1  $\mu$ g/ml) was used as positive control. nt, untreated negative control; The data are mean values (+ SD) of 4 independent experiments; \*\*\* $p$ <0.001. (B) HEK-TLR7HA cells were transfected with 3  $\mu$ g/ml siRNA used in panel A tagged with biotin and with or without 3  $\mu$ g/ml unlabelled inhibitory (inh.) or control (ctr.) RNA. The cells were lysed and streptavidin or anti-HA agarose beads were added for 3 h. The samples were washed and western blot analysis was performed using an anti-HA antibody. The data are from one experiment representative of five.

Next, we wanted to be sure that the inhibitory RNA not only blocks the binding of stimulatory RNA to the receptor, but binds directly to it. The approaches tested are shown in Figure 5-18A. In a first approach, biotinylated tRNA<sup>Tyr</sup> with or without methylation at position 18 was used for co-immunoprecipitation of TLR7-HA. The protein bound to the pulled-down tRNA<sup>Tyr</sup> was analysed by western blotting. For the methylated tRNA and for the unmodified tRNA, slight bands could be seen, indicating that both tRNAs were able to bind to the receptor (Figure 5-18B, “1”, “2”). Again, the band detected was not of the expected size. Thus, it could not be excluded that the band detected was an unspecific signal. In a second approach, cells were co-transfected with equal amounts of biotinylated modified tRNA<sup>Tyr</sup> and unlabelled unmodified tRNA<sup>Tyr</sup>. After pull-down of biotinylated RNA with streptavidin beads, a strong signal was detected for TLR7 in the co-immunoprecipitation, comparable to that observed for the biotinylated tRNA<sup>Tyr</sup> alone (Figure 5-18B, “3”). By contrast, when cells were co-transfected with unmodified biotinylated tRNA<sup>Tyr</sup> plus 2'O-methylated unlabelled tRNA<sup>Tyr</sup>, only a weak band for TLR7 could be detected in the co-immunoprecipitation (Figure 5-16B, “4”).

The assays used were somewhat limited in quality and interpretation. The biotinylated RNA had to reach the endosome and bind to the receptor. Thus, the amount of TLR7 in the endosomes was limited. Moreover, we could not be certain that all biotinylated RNAs were introduced by transfection and located in the endosome when the cells were lysed. Not all liposomes containing RNA are taken up at the same time, and RNA in the endosomes would be degraded after a time. Thus, a time point had to be used at which the amount of receptor-bound RNA was maximal. This is perhaps why only weak bands could be detected.

Together, these preliminary data indicate that unmethylated and methylated tRNA<sup>Tyr</sup> might compete for binding at the same receptor and that methylated tRNA<sup>Tyr</sup> binds to TLR7 with a higher affinity than its unmethylated counterpart.

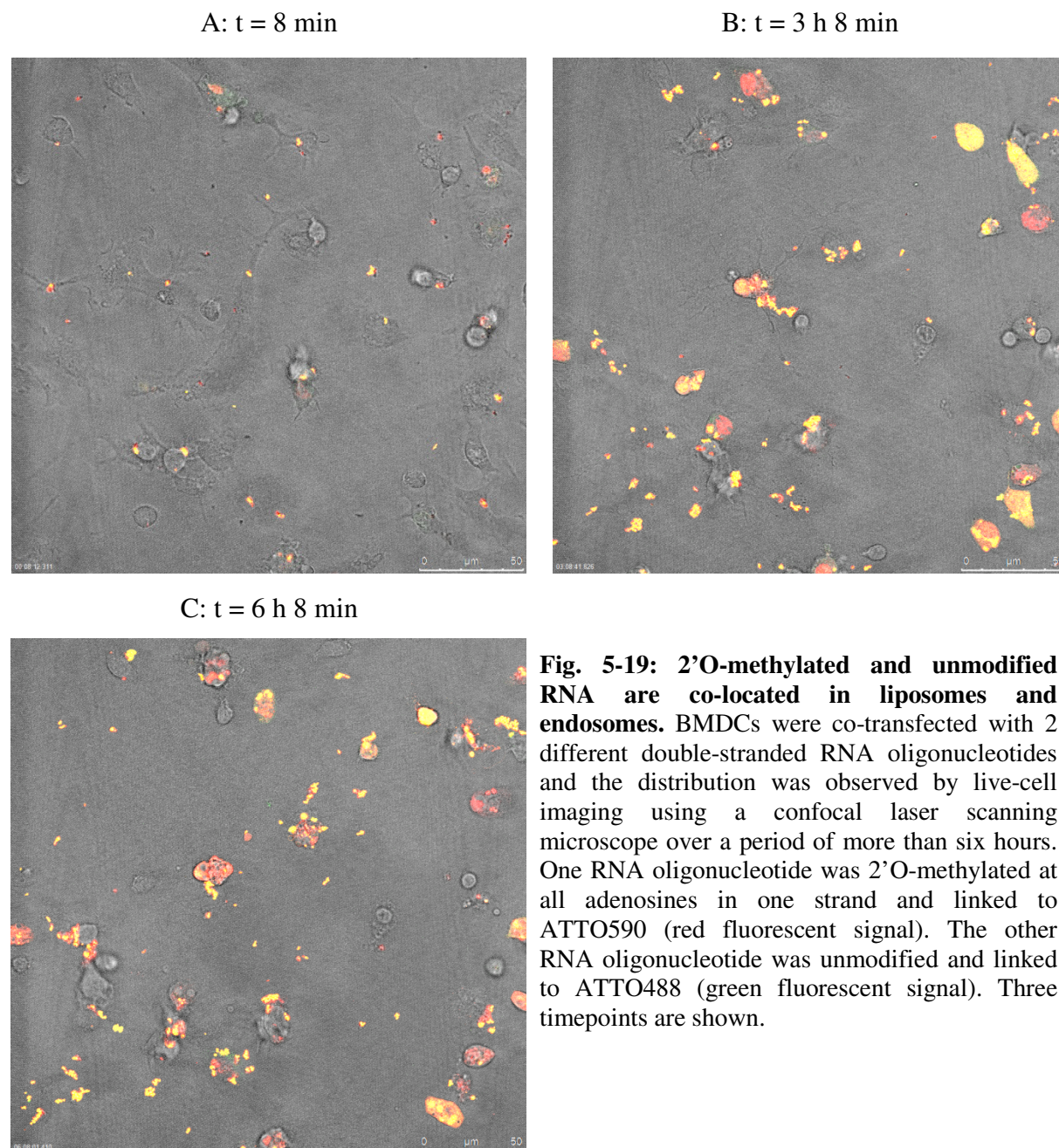


**Fig. 5-18: 2′O-methylated RNA may bind directly to TLR7 and displace unmodified RNA.** (A) Schematic illustration of lysates used for co-immunoprecipitation in panel B. (B) HEK-TLR7HA cells were transfected with 3 μg/ml tRNA tagged with biotin, with or without 3 μg/ml tRNA without any tag. The cells were lysed and streptavidin beads were added for 3 h. The samples were washed and western blot analysis was performed using an anti-HA antibody. The numbers below the blot indicate the tRNA composition illustrated in panel A.

### 5.1.5 Distribution of 2′O-methylated and unmodified RNA in immune cells

In the experiments performed thus far, the stimulatory RNA was mostly introduced by co-transfection with the inhibitory or control RNA. The results indicated that modified and unmodified RNA compete for receptor binding. Thus, both kinds of RNA probably had to be taken up in the same endosomes where TLR7 or TLR8 were expressed. To study this in more detail, murine bone-marrow derived DCs (BMDCs) were co-transfected with two double-stranded synthetic RNA oligonucleotides. They were selected because double-stranded RNA is more stable than single-stranded, and by modifying only one strand the location of the modified strand and the unmodified strand in the same compartment would be ensured. One of the strands was 2′O-methylated at all adenosine residues and linked to the fluorescent dye ATTO590. The other oligonucleotide was the corresponding unmodified RNA

oligonucleotide linked to ATTO488. The sequences of the RNA oligonucleotides are shown in Table 5-2. The distribution of the RNAs was observed by live-cell imaging using a confocal laser scanning microscope over a period of six hours. Pictures at different positions were taken at one-minute intervals.



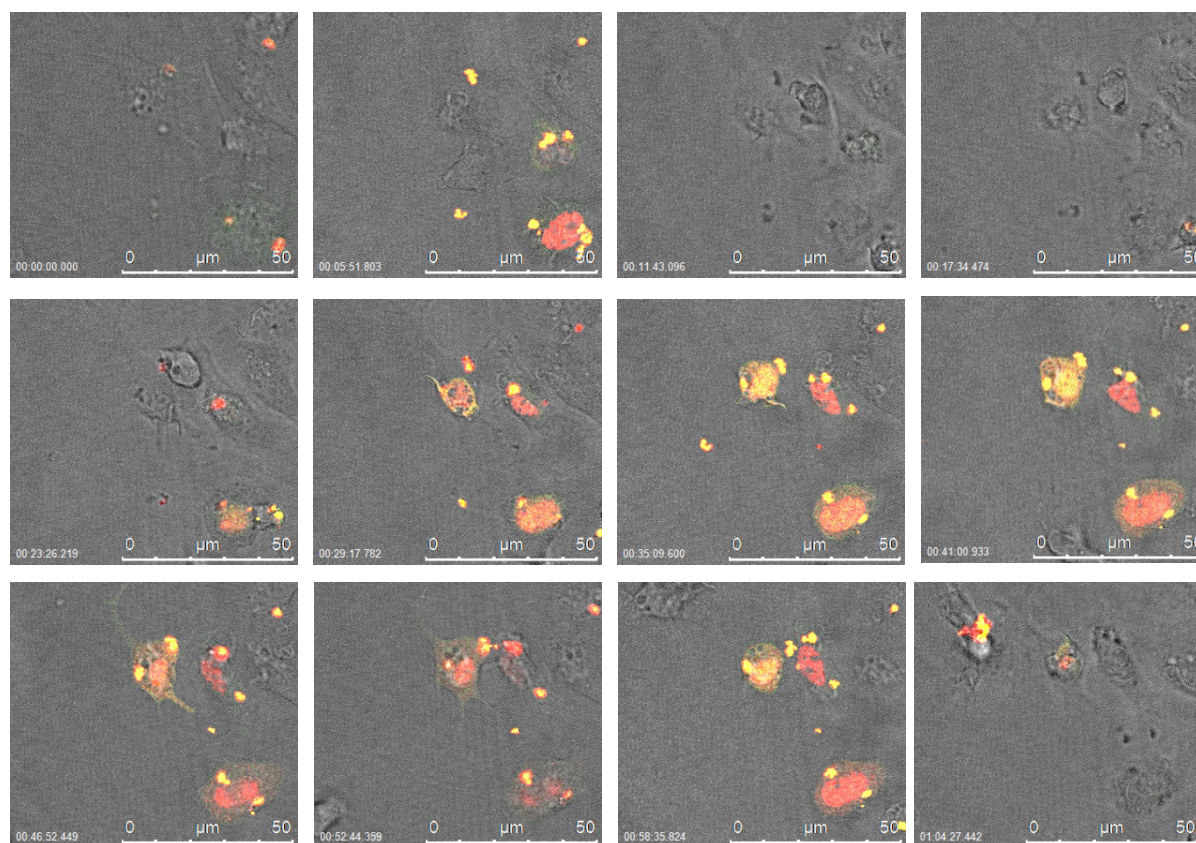
In Figure 5-19, three pictures from different time points are shown. For all time points observed, co-localisation of the different labelled RNAs - indicated by a yellow signal - in small dot-like structures was seen. This indicated a co-localisation in liposomes and endosomes. Also, the efficiency of delivery was calculated using the microscopy data. It was determined to be between 60% and 70% in the different positions and experiments.



**Table 5-2: Sequences of RNA oligonucleotides used in localisation studies**

|    | unmodified RNA (5'→3')          | 2'O-methylated RNA (5'→3')          |
|----|---------------------------------|-------------------------------------|
| s  | GCAAGCUGACCCUGAAGUUCAU          | GCAAGCUGACCCUGAAGUUCAU              |
| as | (ATTO488)GAACUUCAGGGUCAGCUUGCCG | (ATTO590)GAmAmCUUCAmGGGUCAmGCUUGCCG |

In the images, release of the fluorescent-labelled RNA into the cytosol was apparent and different distribution of 2'O-methylated RNA and the unmodified RNA was observed thereafter. This release was observed periodically in all transfected cells. The distribution in the cells was the same each time: the ATTO488-labelled unmodified RNA was mainly located in the cytoplasm, while the 2'O-methylated RNA labelled with ATTO590 was distributed in the nucleus.



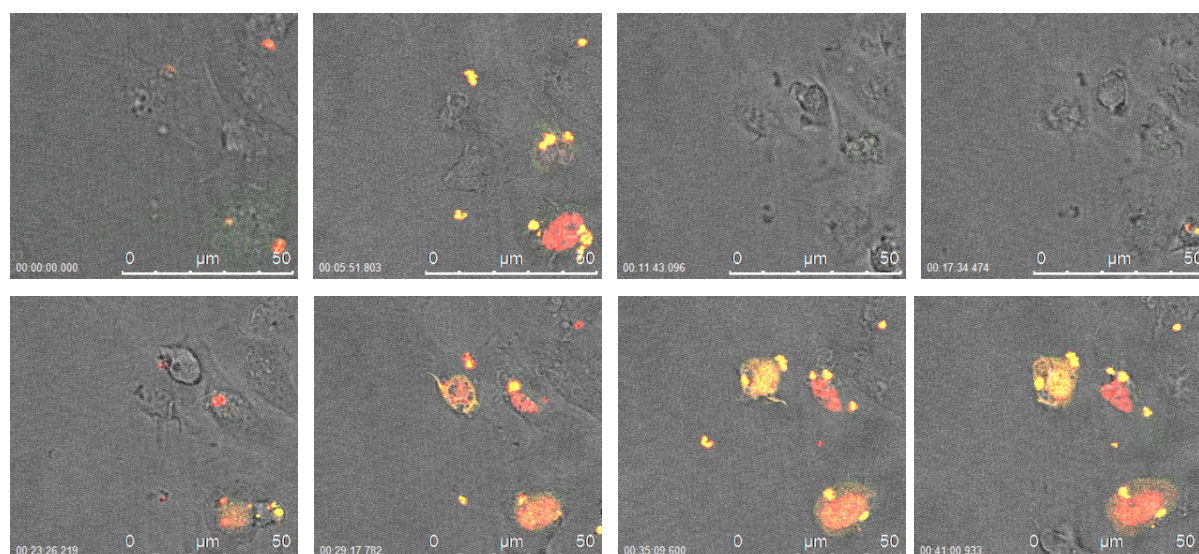
**Fig. 5-20: ATTO488-labelled unmodified RNA oligonucleotides appeared to be co-located with ATTO590-labelled 2'O-methylated RNA oligonucleotides in liposomes and endosomes.** BMDCs were co-transfected with 2 different double-stranded RNA oligonucleotides and the distribution was observed by live-cell imaging using a confocal laser scanning microscope over a period of six hours. One RNA oligonucleotide was 2'O-methylated at all adenosines in one strand and linked to ATTO590 (red fluorescent signal). The other RNA oligonucleotide was unmodified and linked to ATTO488 (green fluorescent signal).

To investigate the distribution in more detail, a representative part of the picture was enlarged and a sequence over one hour is shown in Figure 5-20. Right at the beginning of the observation, the distribution of the fluorescent-labelled RNA showed dot-like structures within the cells and outside of them, consistent with localisation of RNA in liposomes and endosomes after uptake into the cells. The distribution appeared similar for both fluorescent signals. Then, fluorescent-labelled RNA was released into the cytoplasm, resulting in distinct locations of the different RNAs. The red fluorescent signal appeared to localise more in the nucleus whereas the green fluorescence was detected more in the cytoplasm. Still, yellow fluorescent dots-like structures could be seen inside the cell and outside, indicating vesicles containing red and green fluorescent signals. After a while, the fluorescent signals for both RNAs vanished, and recurred later, still co-located in vesicles. During the whole six-hour period, this cycle of dot-like structures and fluorescent RNA released into the cytoplasm with different distributions for both RNAs was consistently observed.

These observations could indicate that both RNAs are co-located in liposomes and endosomes but that they are released from the endosomes and distributed differently inside the cell. The unmodified RNA oligonucleotide was located more in the cytoplasm while the 2'O-methylated RNA oligonucleotide was distributed more in the nucleus. To verify this observation, the experiments were done with the dyes exchanged. The unmodified RNA oligonucleotide was linked to ATTO488 and the 2'O-methylated RNA oligonucleotide was linked to ATTO590. Again the RNAs were used for co-transfection and the cells were observed by live-cell imaging using a confocal laser scanning microscope over a period of six hours. For the transfected cells, a representative part of one position was enlarged and it is shown over a period of 45 min in Figure 5-21. Again, dot-like structures could be seen, indicating co-localisation of the 2'O-methylated and unmethylated RNA in endosomes and liposomes. Again, the release from the endosomes was apparent, followed by different localisation inside the cell. The red fluorescent signal was mainly detected in the nucleus and the green fluorescent signal was mainly detected in the cytoplasm. In this set of experiments, the green fluorescent signal seemed to give a stronger signal than the earlier one.

The data therefore indicated that the different distributions of the 2'O-methylated and unmodified RNAs observed by microscopy were independent of the methylation. Instead, the distribution was due to the different fluorescent dyes used in the experiments.



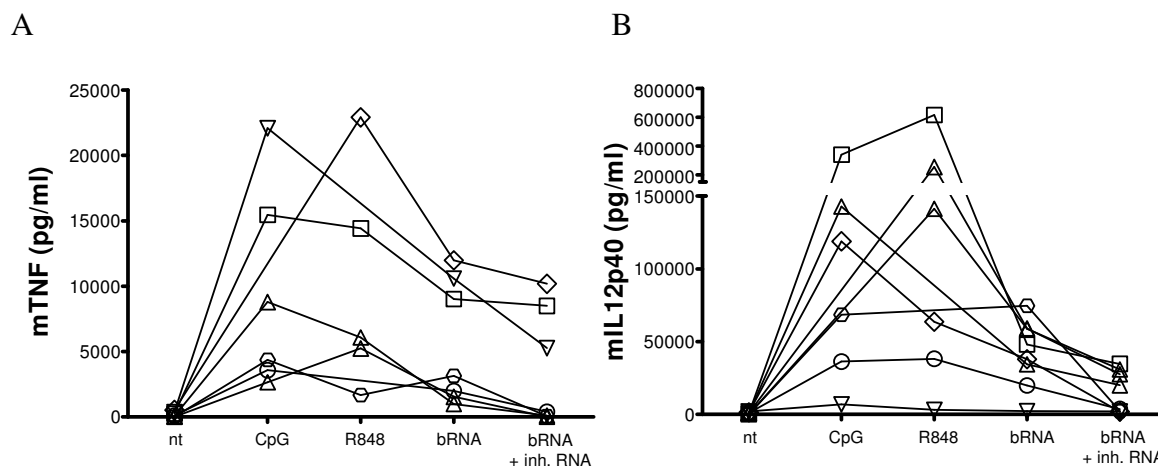


**Fig. 5-21: The distributions of fluorescent labelled RNA oligonucleotides inside the cell were a result of fluorescent dyes used, not the modification.** BMDCs were co-transfected with 2 different double-stranded RNA oligonucleotides and the distributions were determined by live-cell imaging using a confocal laser scanning microscope over a period of six hours. One RNA oligonucleotide was 2'-O-methylated at all adenosines in one strand and linked to ATTO488 (green fluorescent signal). The other RNA oligonucleotide was unmodified and linked to ATTO590 (red fluorescent signal).

### 5.1.6 Inhibitory RNA in the murine system

In the human immune system, bRNA stimulates TLR7 in pDCs to induce IFN- $\alpha$  production and eventually TLR8 in monocytes. In the murine system, TLR8 has been reported to be non-functional. Furthermore, it has been shown that TLR13 is responsible for recognition of bRNA in mice<sup>35,36</sup>.

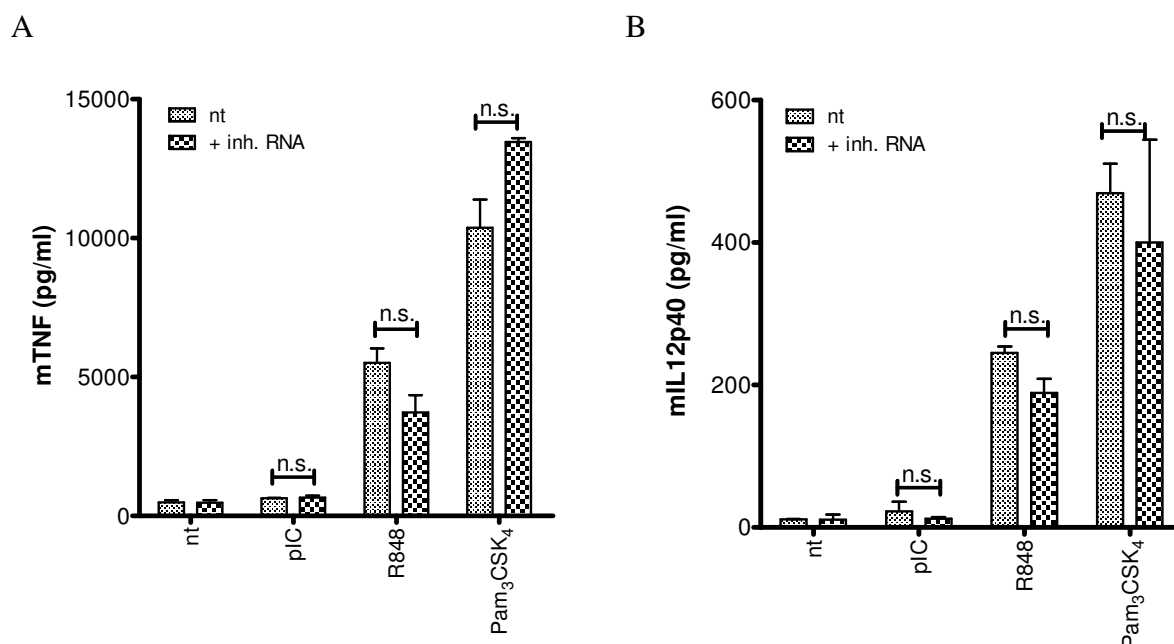
As there are differences between the murine and human systems, we wanted to check whether the inhibitory RNA identified is able to abrogate stimulation by RNA in murine cells. Therefore, murine BMDCs were transfected with bRNA and inhibitory RNA, and production of different cytokines was analysed (Figure 5-22A and B). Secretion of TNF and IL12p40 was induced by bRNA. The effect of the 2'-O-methylated RNA used in the co-transfections was not consistent. While in some experiments the induction of cytokine secretion was inhibited, in other experiments only a slight decrease or even no difference was observed (Figure 5-22A and B, respectively). Interestingly, in the experiments showing a slight decrease in cytokine secretion with inhibitory RNA, bRNA and positive controls induced higher secretion of cytokine compared to the other experiments. The different results may have been due to differences in cell differentiation from using different batches of differentiation medium.



**Fig. 5-22: 2'O-methylated RNA is unable to inhibit cytokine secretion consistently in murine immune cells.** Murine BMDCs were transfected with 1  $\mu\text{g/ml}$  bRNA with or without 2  $\mu\text{g/ml}$  inhibitory RNA. After incubation overnight, cell-free supernatants were analysed for production of (A) TNF and (B) IL12p40. Each symbol represents the mean of one experiment. The experiments were performed using different batches of differentiation medium. nt, untreated negative control. R848 (1  $\mu\text{g/ml}$ ) and CpG-DNA (1  $\mu\text{M}$ ) were used as positive controls.

Further analysis indicated a similar mechanism for the partially observed inhibition as for the human system; the inhibitory effect could be observed at the mRNA level and at the level of MAPK activation. However, the MAPK signal was not entirely abolished, possibly explaining the less efficient inhibition in some experiments. The differences might be due to the expression levels of TLR13. In the cells in which only a slight inhibition could be found, the expression of TLR7 might be reduced while the TLR13 expression was enhanced. In this way, the activation of TLR13 would be responsible for the production of cytokines and the activation would not be inhibited by the 2'O-methylated RNA added.

Again, we investigated whether the effects of other TLR ligands could be inhibited. Inhibition of different TLR ligands would indicate an effect specific for TLRs and exclude a specific effect of RNA stimulation. To test this, different TLR ligands were used to transfect cells along with inhibitory RNA and the cells were examined for cytokine production. The results for TNF and IL12p40 are shown in Figure 5-23. In previous experiments, the different ligands were titrated to avoid concentrations in the range of saturation. For polyI:C, a TLR3 ligand, no induction was observed for both cytokines - even for stimulation without any inhibitory RNA. Both R848 - a TLR7 ligand - and Pam<sub>3</sub>CSK<sub>4</sub> (TLR1/2) led to induction of TNF and IL12p40, which was not significantly affected by co-administration of the inhibitory RNA.



**Fig. 5-23: Inhibitory RNA is unable to inhibit induction of cytokine production by synthetic TLR ligands.** BMDCs were stimulated with different TLR ligands mixed with (dotted bars) or without (grey bars) 2  $\mu$ g/ml inhibitory RNA (inh. RNA) before encapsulation in lipoplexes. The cells were incubated overnight and cell-free supernatants were examined for (A) TNF and (B) IL12p40 production, using ELISA. R848 (TLR7): 20 ng/ml; pIC (TLR3): 1  $\mu$ g/ml; Pam<sub>3</sub>CSK<sub>4</sub>(TLR1/2): 1  $\mu$ g/ml. nt, untreated negative control; The data are mean values (+ SD) of duplicates in one experiment representative of three.

### 5.1.7 Summary of part 5.1

So far, we had found that RNA containing a 2'-O-methyl modification at position G18 - as occurs naturally in *E. coli* tRNA<sup>Tyr</sup> - could inhibit the production of IFN- $\alpha$  induced through TLR7 activation in human pDCs by stimulatory RNA. We also found that 2'-O-methylated RNA is also capable of inhibiting TNF, IL12p40, and IL6 production by human monocytes in response to stimulatory RNA. Inhibition occurred at the level of mRNA expression without any time lag. Early events in signal transduction were inhibited. Both the stimulatory RNA and the inhibitory RNA appeared to bind directly to the receptor, and this binding was competitive. Inhibitory RNA displaced the stimulatory RNA from the receptor, and probably had higher affinity. The specificity of inhibitory RNA for inhibition of stimulation by bRNA was proven by lack of inhibition of CpG-DNA (TLR9) and Pam<sub>3</sub>CSK<sub>4</sub> (TLR1/2). Interestingly, stimulation of TLR7 and TLR8 by R848 could also not be inhibited by 2'-O-methylated RNA, indicating that there may have been a different mode of ligand recognition for R848 than for bRNA.

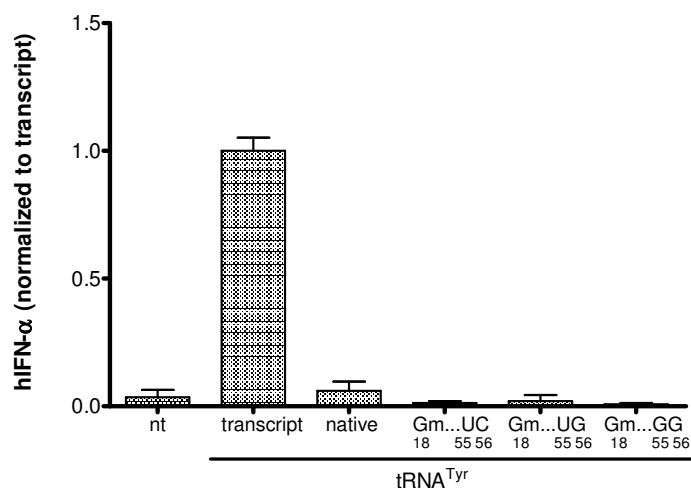
Murine BMDCs showed only minor inhibitory effects of 2'-O-methylated RNA, indicating that the mouse-specific TLR13 may have an important role that is not affected by the presence of 2'-O-methylated RNA.

## 5.2 Identification of a 2'-O-methylated dinucleotide motif responsible for tRNA silencing

It is assumed that TLR7 recognises RNA depending on a certain order of nucleotides<sup>118</sup>, but the exact stimulatory motif is still poorly defined. It was discovered very recently that post-transcriptional modifications play an important role in this context. In native tRNA<sup>Tyr</sup>, there is a methylation of the 2'-hydroxyl function of the ribose of the guanosine at position 18<sup>198</sup>. This guanosine is located in the structural core of the L-shaped tRNA. The primary function of the modification may be structural stabilisation of the region<sup>193,203,204</sup>. The role of this modification in immune stimulation may have evolved as a secondary feature. To better understand the function of the sequence for TLR7 recognition, we produced different modivariants of tRNA<sup>Tyr</sup> that differed regarding the nucleoside that was methylated, the next nucleoside downstream, and the position of the methylation within the tRNA sequence. Modivariants of a tRNA have the same sequence as the native tRNA, and only one or two positions are modified or mutated. They are produced by ligation of three different parts of the tRNA using a DNA template<sup>199</sup>. The different modivariants were tested for their ability to induce cytokine expression in human PBMCs. The production of different cytokines due to transfection with the different RNAs was measured by ELISA.

### 5.2.1 The influence of three-dimensional structure on immunosilencing

As the methylation of tRNA<sup>Tyr</sup> at position 18 is important for structural stability of the region<sup>124</sup>, we studied the influence of the three-dimensional structure on immunosilencing. The native tRNA<sup>Tyr</sup> forms hydrogen bonds between G18 and G19 - with C56 and U55, respectively. The methylation of the ribose at position 18 might influence this structure, thereby leading to immunosilencing. To investigate this possibility, the nucleobases 55 and 56 in the T-loop of tRNA<sup>Tyr</sup> were mutated to guanosines to abrogate the building of hydrogen bridges and thereby destroy the three-dimensional structure of the tRNA (Figure 5-24). Mutations in the T-loop resulted in an unaltered, silent tRNA. Changing of the cytidine at position 56 to guanosine gave the same weak level of cytokine secretion as the native sequence with methylation at position 18. No difference in IFN- $\alpha$  induction was observed when position 55 was also mutated to guanosine. We therefore concluded that the three-dimensional structure had no influence on the immunosilencing efficiency of the methylated tRNA<sup>Tyr</sup>.



**Fig. 5-24: The three-dimensional structure has no influence on immunosilencing capacity of tRNA.**

Different modivariants of tRNA<sup>Tyr</sup> were synthesised and PBMCs were transfected with each at 500 ng/ml. Cell supernatants were assayed for IFN- $\alpha$  production by ELISA. The native tRNA<sup>Tyr</sup> contained all modification that naturally occur in the tRNA, while all other tRNAs contained only Gm18. The numbers under the bases indicate the position in the tRNA. The data are mean values (+SD) from 2 independent donors, each measured in triplicate and normalised

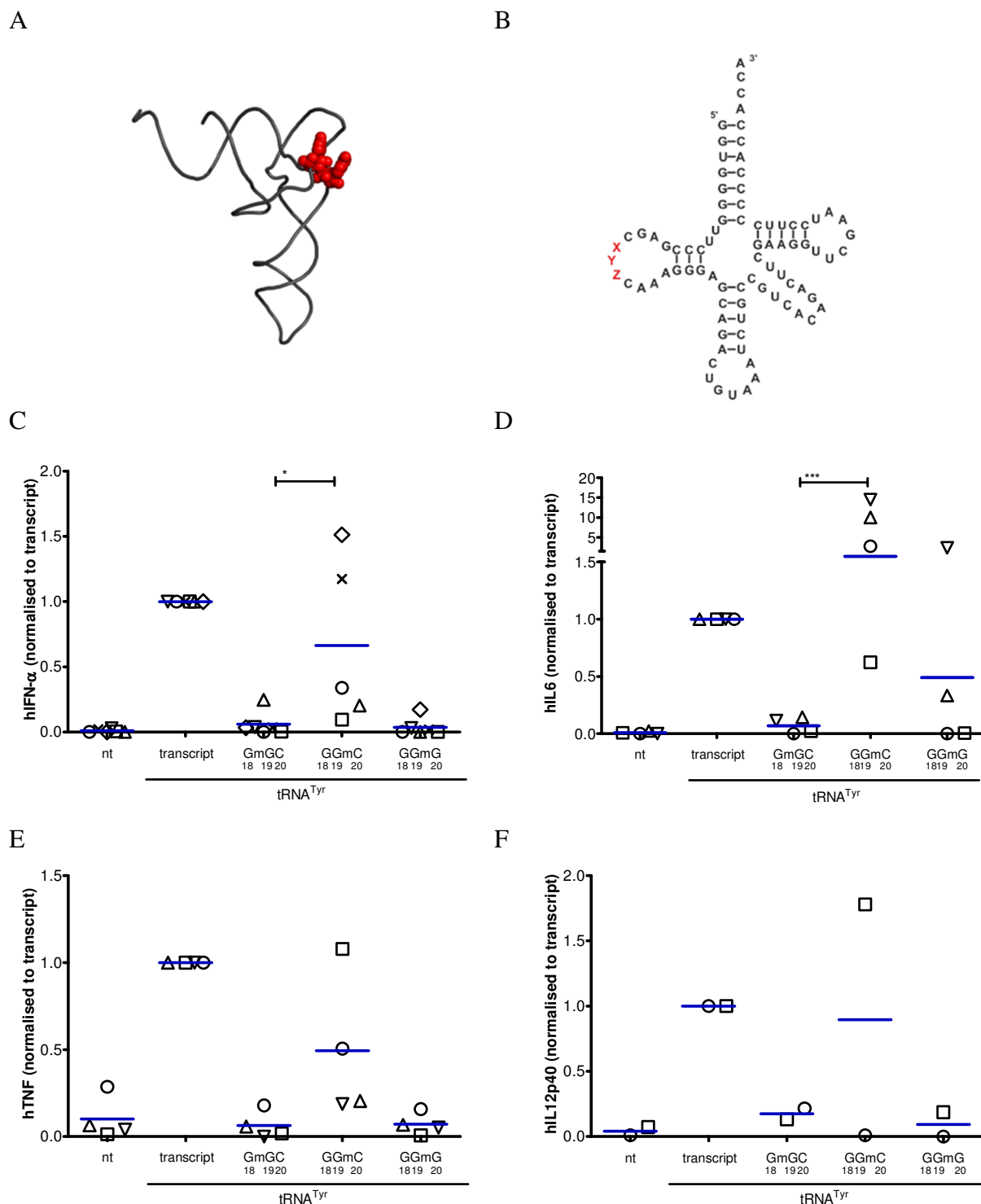
to the unmodified *in vitro* transcript of tRNA<sup>Tyr</sup> to avoid donor-related variation. nt, untreated negative control.

### 5.2.2 Methylation of guanosine alone is not sufficient for immunosilencing

Native tRNA<sup>Tyr</sup> has a ribose-methylated guanosine at position 18. This is followed by another guanosine (GmG). To study position-dependent effects of this inhibitory modification, we shifted the methylation downstream by one nucleotide - to position 19. The molecular weight and the sequence of the tRNA were the same, and only the position of the methyl group differed by 5 Å. The resulting tRNA was tested for induction of cytokine expression. The positions of the 2'O-methylations in the three-dimensional structure and the sequence are shown in Figure 5-25A and Figure 5-25B, respectively.

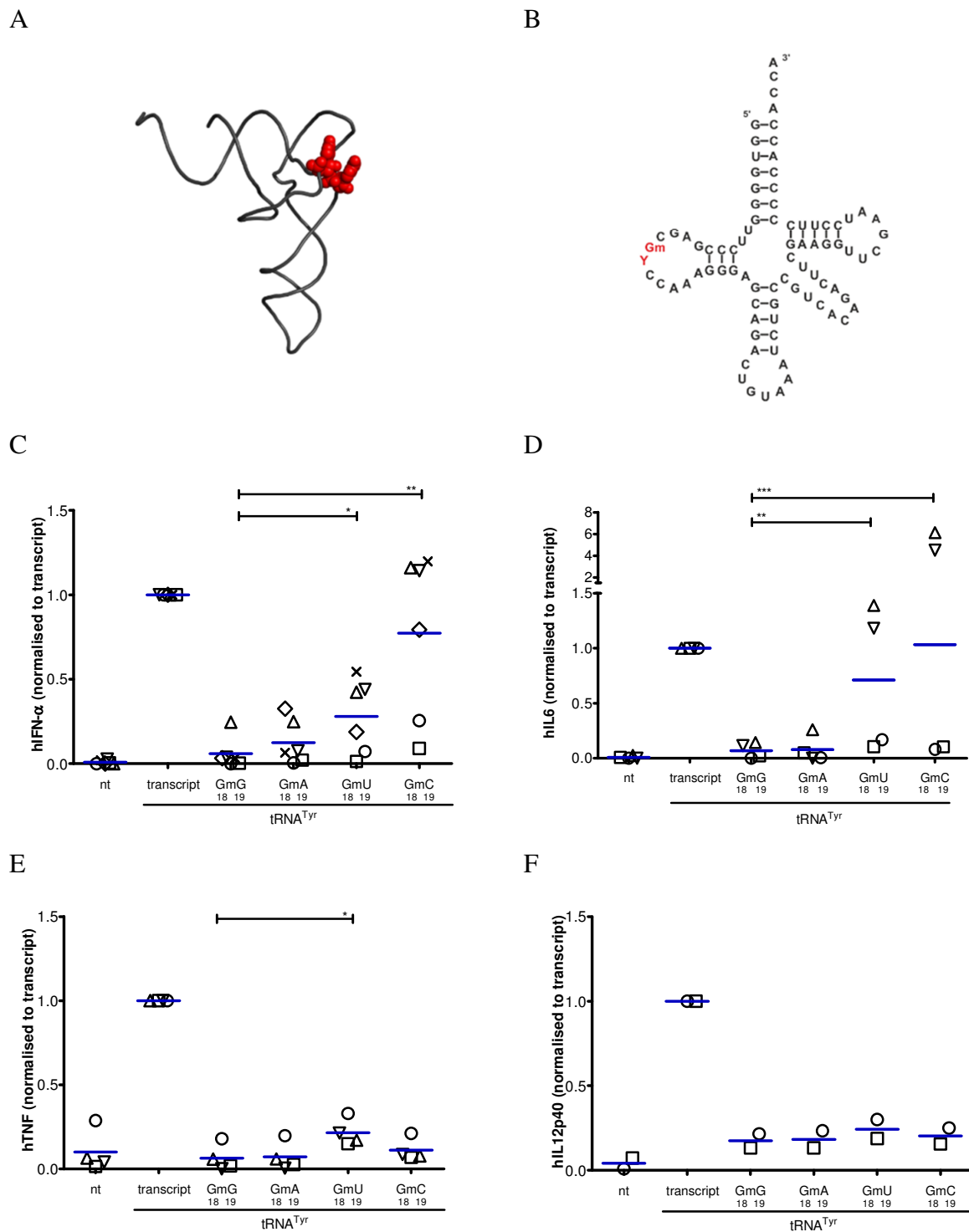
Analysis of Figure 5-25C–F shows that the immunosilencing ability of the tRNA was negatively affected in this new construct for all cytokines tested. While the modification at the original position (Gm18) prevented induction of cytokine production, moving of the methylation to position 19 resulted in high levels of cytokine secretion. The exact degree of induction depended on the cytokine and the donor, but in all cases the induction was significant. For IFN- $\alpha$  and TNF production, most donors showed only slight induction and only one or two donors showed an increase comparable to that with the transcript alone. For IL6, most of the donors led to an increase up to 15 times higher than that for the transcript.

Analysis of the motif around the modification showed that the former motif GmG was changed by shifting to methylation to position 19, resulting in GmC. Interestingly, the immunosilencing effect was completely recovered by changing the nucleobase at position 20 to guanosine. The resulting motif GmG was now the same as for methylation at position 18, indicating that the sequence motif played an important role in immunosilencing.



**Fig. 5-25: translocation of the methylated motif by one nucleotide downstream affects immunosilencing.** (A) Location of the nucleotides 18 and 19 in the three-dimensional model of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (B) Location of nucleotides 18 (X), 19 (Y), and 20 (Z) in the sequence of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (C-F) PBMCs of two to six donors were activated by transfection with the indicated tRNA (500 ng/ml) overnight. The methylation at position 18 (GmGC) was translocated to position 19 (GGmC) and the nucleobase at position 20 was changed from cytidine to guanosine (GGmG). Cell-free supernatants were used to measure production of (C) IFN- $\alpha$ , (D) IL6, (E) TNF, and (F) IL12p40 by ELISA. Data were normalised to the unmodified tRNA transcript to avoid donor dependency. Each dot in the figure represents the mean of duplicates from one donor, and blue lines represent the mean of all donors. nt, untreated control; \* $p < 0.05$ ; \*\*\* $p < 0.001$

To examine the influence of all nucleobases on the immunosilencing effect, other mutants of the tRNA were synthesised. These tRNAs included the ribose methylation at position 18, while the identity of the nucleoside at position 19 varied. The location of the transposed base in the three-dimensional structure and in the sequence are shown in Figure 5-26A and Figure 5-26B, respectively. The cytokine induction capacity of the different variants is shown in Figure 5-26C–F. For all cytokines, the native sequence GmG failed to cause immunostimulation. Changing of the guanosine at position 19 to adenosine did not alter expression of any cytokine. Changing of the guanosine at position 19 to a uridine led to a significant increase in expression of all cytokines except for IL12p40. For IFN- $\alpha$  and IL6, the strongest increase in cytokine secretion was induced by a tRNA with a cytidine at position 19. For TNF, the production was also significantly higher than with the native sequence GmG, but less than with GmU. For IL12p40 induction, no significant differences were observed for all sequence motifs tested. The sequence motifs with their corresponding cytokine induction are summarised in Table 5-3. Interestingly, the sequence motif GmC was able to induce high amounts of TNF when G19 was 2'-O-methylated, while no TNF induction was observed with the 2'-O-methylated G18 followed by a cytidine. For the other cytokines, no differences between the two methylation sites were observed. This indicated that the nucleobase following the methylated position had a greater influence on the immunosilencing effect on IFN- $\alpha$  and IL6 production, while for silencing of TNF and IL12p40 the nucleobase appeared to have no effect. This might be due to different specificities of TLR7 and TLR8.



**Fig. 5-26: The nucleobase following at position 19 influences the immunosilencing effect.** (A) Location of nucleotides 18 and 19 in the three-dimensional model of tRNA<sup>Tyr</sup> (adapted from AG Helm, Mainz). (B) Location of nucleotides 18 (Gm) and 19 (Y) in sequence of tRNA<sup>Tyr</sup> (adapted from AG Helm, Mainz). (C-F) PBMCs from two to six donors were activated by transfection with the indicated tRNA (500 ng/ml) overnight. The different tRNAs had a 2'-O-methylation at position 18 and were mutated at the next position to different nucleobases. Cell-free supernatants were used to measure production of (C) IFN- $\alpha$ , (D) IL6, (E) TNF, and (F) IL12p40 by ELISA. Data were normalised to the unmodified tRNA transcript to avoid donor dependency. Each dot in the figure represents the mean of duplicates from one donor, and the blue lines represent the mean of all donors. nt, untreated negative control; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$



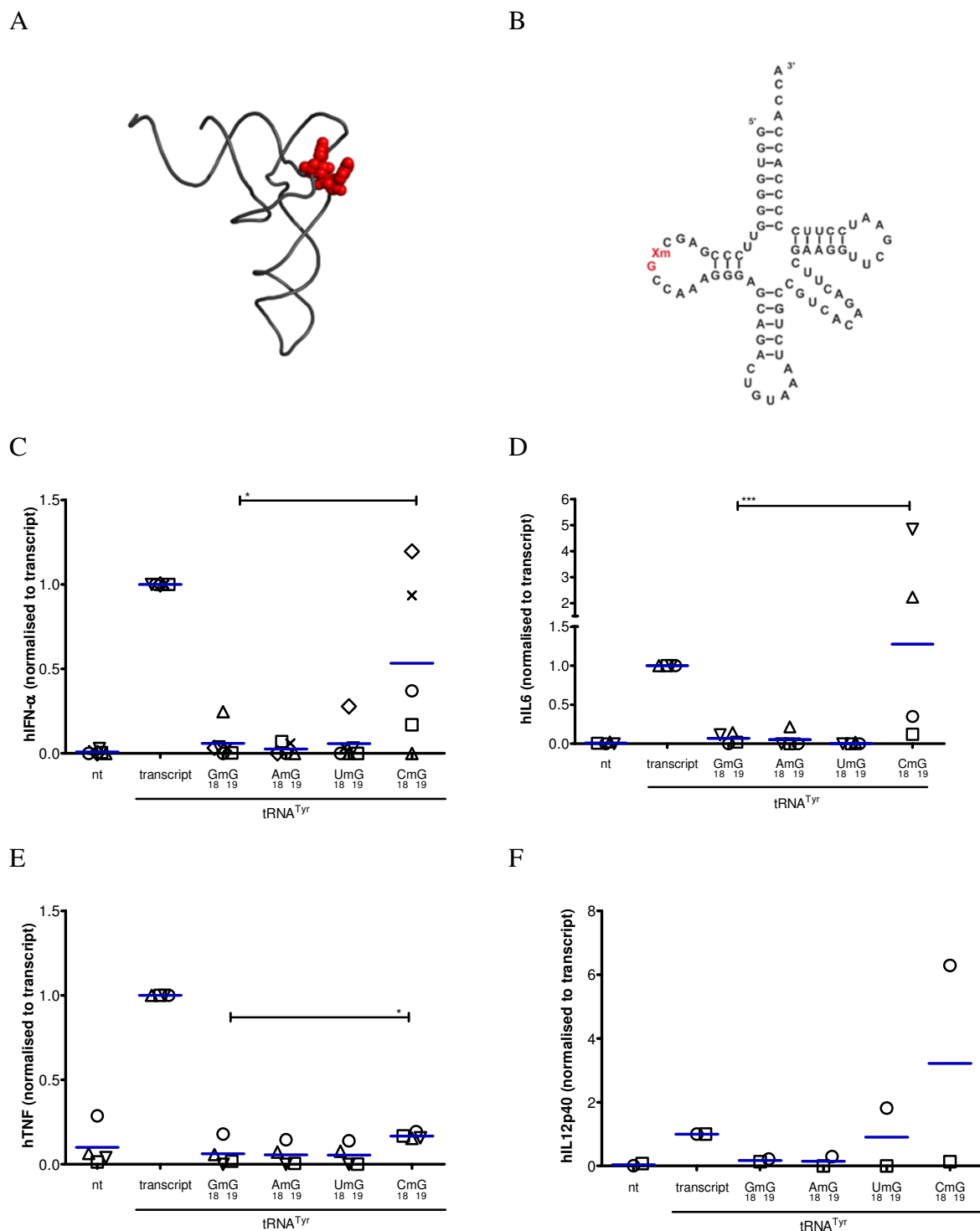
Taken together, the data indicated that the ribose methylation at position 18 alone was not sufficient to abrogate induction of IFN- $\alpha$  and IL6. The immunosilencing effect was highly efficient when a purine (adenosine or guanosine) was present at position +1. The presence of a pyrimidine (uridine or cytidine) had an adverse effect on the silencing. This was less strong for uridine but stronger for cytidine. In the natural context, the sequence at position 18 of tRNAs is highly conserved. Especially in species expressing TrmH, the enzyme responsible for Gm18 in tRNAs, the identified sequence context around G18 is widely spread among different species. This highlights the relevance of our findings, as they correspond to the usual situation in tRNAs<sup>194</sup>.

**Table 5-3: Stimulation of cytokine expression by different tRNAs containing diverse sequence motifs around the 2'O-methylation.**

|     | <b>IFN-<math>\alpha</math></b> | <b>IL6</b> | <b>TNF</b> | <b>IL12p40</b> |
|-----|--------------------------------|------------|------------|----------------|
| GG  | +++                            | +++        | +++        | +++            |
| GmG | -                              | -          | -          | -              |
| GmA | +                              | -          | -          | -              |
| GmU | ++                             | +          | +          | -              |
| GmC | +++                            | ++         | -/+++      | -              |

### 5.2.3 Permutation of the 2'O-methylated base

In the next step, we studied the influence of the nucleobase itself at the methylated ribose. Again, different variants of the tRNA - differing at the methylated position 18 - were synthesised. The location of the changed nucleobase is shown in Figure 5-27A and B. The induction of the different cytokines is shown in Figure 5-27C-F. As expected, the native sequence (GmG) showed no induction of any cytokine. Mutation of guanosine at position 18 to adenosine again showed no cytokine induction; nor did mutation of position 18 to uridine. However, a cytidine at the methylated position 18 led to induction of all cytokines. This induction was significant - compared to the native nucleobase Gm - for all cytokines except of IL12p40. Here, great donor variation prevented any exact conclusion.



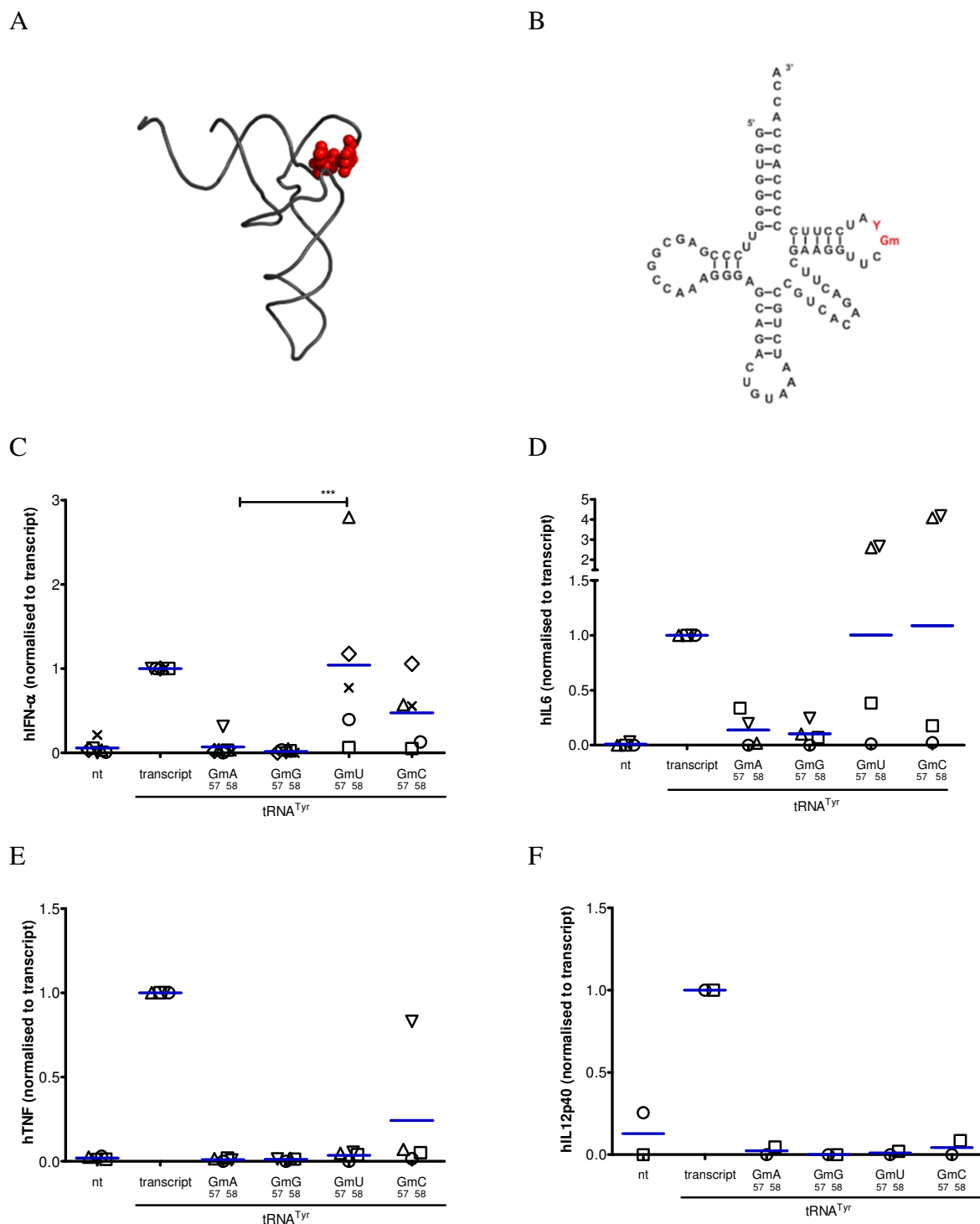
**Fig. 5-27: Cytidine with a methylated ribose lacks immunosilencing activity.** (A) Location of nucleotides 18 and 19 in the three-dimensional model of tRNA<sup>Tyr</sup> (adapted from AG Helm, Mainz). (B) Location of nucleotides 18 (Xm) and 19 (G) in the sequence of tRNA<sup>Tyr</sup> (adapted from AG Helm, Mainz). (C-F) PBMCs from two to four donors were activated by transfection with the indicated tRNA (500 ng/ml) overnight. The different tRNAs had a 2'-O-methylation at position 18 and were mutated at the same position, to different nucleobases. Cell-free supernatants were used to measure production of (C) IFN- $\alpha$ , (D) IL6, (E) TNF, and (F) IL12p40 by ELISA. Data were normalised to the unmodified tRNA transcript to avoid donor dependency. Each dot in the figure represents the mean of duplicates from one donor, and the blue lines represent the mean of all donors. nt, untreated negative control; \* $p < 0.05$ ; \*\*\* $p < 0.001$

In summary, these results indicated that the nucleobase itself was less important for lack of induction, as long as it was not cytidine. All variants tested, including a methylated ribose and a downstream purine, led to efficient immunosilencing. The only exception was cytidine. Compared to the unmethylated tRNA, 2'-O-methylated cytidine at position 18 did lead to silencing but less efficiently than the other nucleosides, and with strong donor variability (Figure 5-27). We therefore believe that the functional motif for immunosilencing is DmR, where D is any nucleobase except cytosine and R is a purine. This dinucleotide motif appears to be necessary for silencing of the induction of IFN- $\alpha$  and IL6 expression, but not for silencing of induction of TNF and IL12p40 expression, where the 2'-O-methylation itself appears to suffice. This might indicate different motif specificities for TLR7 and TLR8.

#### **5.2.4 Location of the 2'-O-methylated nucleobase in tRNA is not crucial for abrogation of stimulation**

In the experiments above, the 2'-O-methylation was always present in the D-loop of tRNA<sup>Tyr</sup>. Only the methylated nucleoside itself or the nucleoside +1 upstream of the methylation was permuted.

Next, we wanted to know whether the methylated motif could be transposed to a completely different structural context within the same tRNA, and thereby keeping its characteristics to act immunosilent. To test this, the methyl group was transposed to two other loop regions of the tRNA, at position 34 and position 57 in the anticodon loop and T-loop, respectively. Methylation of G34 occurs naturally in eukaryotic tRNA<sup>Phe</sup><sup>194</sup> while methylation at G57 has not been detected so far in native tRNAs. The nucleobase following the methylation was mutated to determine whether the sequence motif for immunosilencing was the same at different positions inside the tRNA.



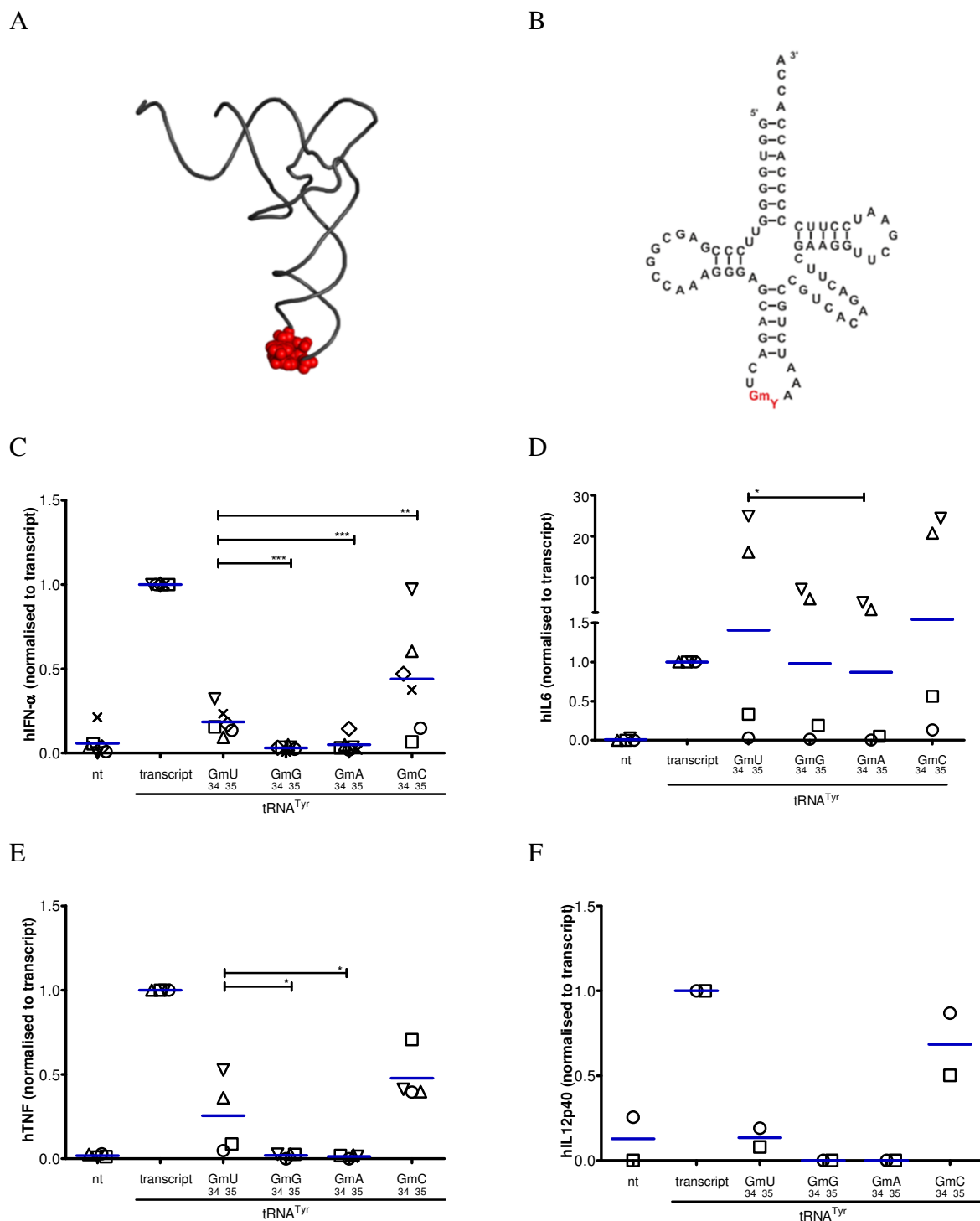
**Fig. 5-28: Methylations in the T-loop showed the same sequence preference for immunosilencing.** (A) Location of nucleotides 56 and 57 in the three-dimensional model of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (B) Location of nucleotides 56 (Gm) and 57(Y) in the sequence of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (C-F) PBMCs from two to five donors were activated by transfection with the indicated tRNA (500 ng/ml) overnight. The different tRNAs contained a 2'-O-methylation at position 57, and position 58 was mutated to different nucleobases. Cell-free supernatants were used to measure production of (C) IFN- $\alpha$ , (D) IL6, (E) TNF, and (F) IL12p40 by ELISA. Data were normalised to the unmodified tRNA transcript to avoid donor dependency. Each dot in the figure represents the mean of duplicates from one donor, and the blue lines represent the mean of all donors. nt, untreated negative control; \*\*\* $p < 0.001$

For the transposition of the methyl group to position 57 in the tRNA, the new location in the three-dimensional structure and the sequence is shown in Figure 5-28A and Figure 5-28B, respectively. The induction of cytokines by the different tRNA variants is shown in Figure 5-28C–F. In the native context of the tRNA, Gm57 was followed by an adenosine and led to no or only slight induction of cytokine secretion for all cytokines tested. Changing of the adenosine at position 58 to a guanosine gave no cytokine induction. Incorporation of a uridine at position 58 increased the secretion of IFN- $\alpha$  and IL6, but only the secretion of IFN- $\alpha$  was significant. For TNF and IL12p40, no induction was observed. Mutation of position 58 to a cytidine led to increased induction of IFN- $\alpha$  and IL6. For TNF, only cells from one donor out of four increased their cytokine production, while no induction was observed for IL12p40.

In summary, the induction of cytokines showed similar patterns to the permutations at position G19, indicating that an immunosilencing motif could be transposed to other regions of the tRNA.

In most species Gm34, a naturally occurring methylation in eukaryotic tRNA<sup>Phe</sup>, is followed by an adenosine. In the tRNA of *E. coli*, a uridine is present at position 35. The location of the methylated sequence motif in the three-dimensional structure and the sequence is shown in Figure 5-29A and B, respectively. The previous results forecast that this tRNA variant would induce some cytokine production. The cytokine induction by Gm34 with different variations at position 35 is shown in Figure 5-29C–F. The native sequence GmU induced cytokine secretion for all cytokines tested, as expected. Mutation of uridine at position 35 to adenosine or guanosine reduced the signal compared to Gm34U. This decrease was significant for both IFN- $\alpha$  and TNF induction, and significant with GmA for IL6 production. The cytokine production was increased for GmC, even though the signals were highly donor-dependent. For IL6, great donor variation was observed. For all donors, GmU and GmC induced higher amounts of the cytokine than GmG and GmA, but cells from two donors had similarly low production for all tRNA variations while cells from the other two produced amounts of cytokines that were around 10 times higher than with the transcript.

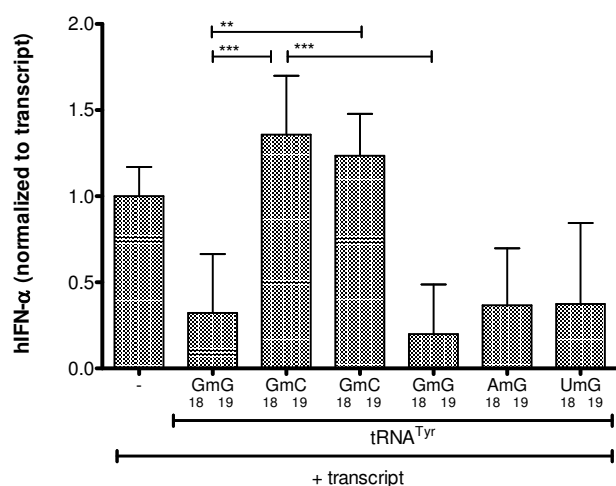
The results supported our previous observations, suggesting that for immunosilencing in the context of IFN- $\alpha$  production, the motif DmR is functional at different positions in a tRNA - and highlighting the relevance of this motif for TLR7 activation in pDCs.



**Fig. 5-29: Methylations in the anticodon loop showed the same preferences for immunosilencing.** (A) Location of nucleotides 34 and 35 in the three-dimensional model of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (B) Location of nucleotides 34 (Gm) and 35(Y) in the sequence of  $tRNA^{Tyr}$  (adapted from AG Helm, Mainz). (C-F) PBMCs from two to five donors were activated by transfection with the indicated tRNA (500 ng/ml) overnight. The different tRNAs contained a 2'-O-methylation at position 34, and position 35 was mutated to different nucleobases. Cell-free supernatants were used to measure production of (C) IFN- $\alpha$ , (D) IL6, (E) TNF, and (F) IL12p40 by ELISA. Data were normalised to the unmodified tRNA transcript to avoid donor dependency. Each dot in the figure represents the mean of duplicates from one donor, and the blue lines represent the mean of all donors. nt, untreated negative control; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

### 5.2.5 Immunoinhibitory potential of the immunosilencing motif

It has been shown that the non-stimulatory effect of native tRNA<sup>Tyr</sup> is paralleled by an additional inhibitory effect on co-transfected stimulatory RNA<sup>123</sup>. To determine whether this effect could be confirmed for other tRNA variants with immunosilencing properties, a selection of the previously tested tRNAs were used for co-transfection along with the unmodified transcript, and IFN- $\alpha$  production was measured by ELISA. The results are shown in Figure 5-30. The first data entry in the bar chart corresponds to where the stimulatory transcript alone was blotted; the stimulation of all other samples were normalised to this. After adding Gm18G and Gm19G the stimulation decreased, indicating an inhibitory effect. The tRNAs containing the motifs Gm18C and Gm19C were stimulatory on their own, and therefore no inhibition was expected. The amount of IFN- $\alpha$  was higher than with the transcript alone. Two other tRNA mutants that showed immunosilencing in Figure 5-24 were tested for immune inhibition. They were able to reduce the IFN- $\alpha$  signal from the transcript. We therefore believe that the motif DmR is not just immunosilent, i.e. is not recognised by the receptor, but antagonises the immune-activation properties of unmodified RNA.



**Fig. 5-30: Immunosilent tRNA mutants are able to inhibit stimulation by transcript.** PBMCs of two to five donors were activated by co-transfection with 500 ng/ml tRNA and *in-vitro* transcript. ELISA was used to measure production of IFN- $\alpha$ . Data represent mean + SD for six independent donors normalised to transcript alone (-); \*\*,  $p < 0.01$

### 5.2.6 Summary of part 5.2

In the previous section, tRNA<sup>Tyr</sup> was mutated at different locations to achieve a better understanding of the motif responsible for immunosilencing.

First, the influence of the three-dimensional structure was analysed. By mutating the nucleosides at position 55 and 56 to guanosines, hydrogen bonding was disrupted and folding of the tRNA was destroyed<sup>205</sup>, but the immunosilencing effect of the methylation was unchanged.

Permutation studies were performed at three locations in the D-loop, the anticodon loop, and the T-loop concerning the nucleobase at the methylated ribose and the 3' downstream nucleobase. The results indicated that a purine base at the position 3' of the methylated ribose leads to the greatest silencing effect. Furthermore, pyrimidines affect the immunosilencing adversely, but cytosine more than uracil. For all stimulatory tRNAs, high donor variations were observed.

Selected tRNAs were further tested for immune inhibition. All the immunosilent tRNAs tested were able to reduce IFN- $\alpha$  production by stimulation with the unmodified, stimulatory transcript.

Thus, a methylated dinucleotide DmR (where D is A, G, or U but not C; R is a purine) could be identified that is responsible for the immunosilencing effect and can also lead to inhibition. This dinucleotide sequence can be found in a wide range of bacterial tRNAs, especially in many *E. coli* tRNAs such as tRNA<sup>Gln</sup>, tRNA<sup>Arg</sup>, and tRNA<sup>Ser</sup>, and also in *B. subtilis* tRNA<sup>Phe</sup> and *S. typhimurium* tRNA<sup>Gly</sup>.



## 6 DISCUSSION

RNA modifications play an important role in innate immunity to distinguish self RNA from foreign RNA. Modifications incorporated into RNA molecules can result in an abrogation of immunostimulation. In this study, *E. coli* tRNA<sup>Tyr</sup> was analysed in detail. It has been shown previously that 2'O-methylation at position G18 is sufficient and necessary for silencing of IFN- $\alpha$  production via TLR7 in pDCs. In this work, it was shown that 2'O-methylated RNA is also capable of inhibiting stimulation by RNAs that would otherwise have activating properties, and in a dominant manner. This dominant inhibitory effect was also shown for production of pro-inflammatory cytokines including TNF and IL12p40 by monocytes. This inhibition had similar efficiency to the inhibition of IFN- $\alpha$ . The inhibitory effect of 2'O-methylated RNA was specific for RNA, as no other TLR ligands were inhibited.

The results suggest a direct binding of the methylated RNA to TLR7, and strong competition with unmodified RNA. Furthermore, co-localisation of modified and unmodified RNA in endosomes was observed by microscopy.

The sequence motif DmR was identified to be important for the immunosilencing effect. Here, D stands for all bases except cytosine and R indicates a purine. This RNA motif was found to be strictly necessary for inhibition of IFN- $\alpha$  and IL6 production, while TNF and IL12p40 production could to be silenced by most of the 2'O-methylated motifs and often resulted in great donor-related variation.

### 6.1 Inhibition by 2'O-methylated RNA

#### 6.1.1 One 2'O-methylation in tRNA<sup>Tyr</sup> is sufficient for the immunosilencing effect

For the first time, this study analysed the immunomodulatory effects of an RNA modification in its natural context, namely that of tRNA. It was found that the methylation at position 18 in *E. coli* tRNA<sup>Tyr</sup> is necessary and sufficient not only for silencing of immune activation, but also to act as a dominant inhibitor, resulting in inhibition of an otherwise stimulatory RNA (Figure 5-2). In other studies using siRNA as the model RNA, at least two positions were 2'O-methylated in a 21-nucleotide RNA strand<sup>187,190</sup>. One study screened for the minimal amount of 2'O-methylation necessary for immunosilencing. In that study, the authors

analysed siRNA strands with 1, 2, 3, or 5 methylations for their immune activation and immunoinhibitory capacity by transfecting PBMCs with them together with a stimulatory RNA strand. The 2'-O-methylations were incorporated at positions with different nucleobases in the siRNA strand. The authors identified a single 2'-O-methylation at adenosine in the siRNA as being immunosilencing and also immunoinhibitory for IFN- $\alpha$  production<sup>206</sup>. This result is similar to the results in our study; only the nucleobase at the 2'-O-methylated position differed.

The gene *trmH* encodes the methyltransferase TrmH, which is responsible for methylation of G18 in bacterial tRNAs. This modification mediates the immunosilencing effect of *E. coli* tRNA preparations<sup>123</sup>. Deletions of other methyltransferases (as done for TrmA or TrmB, incorporating m<sup>5</sup>U54 or m<sup>7</sup>G47, respectively) were unable to modulate the non-stimulatory characteristic of the *E. coli* tRNA preparation<sup>124</sup>. This was analysed by introduction of isolated tRNAs of the deletion mutants into PBMCs and measurement of IFN- $\alpha$ . The tRNA preparations from TrmA and TrmB deletion mutants were unable to induce cytokine production, while the tRNA preparation from a TrmH deletion mutant induced IFN- $\alpha$  production<sup>124</sup>. The gene encoding TrmH could only be found in gram-negative bacteria such as *Salmonella typhimurium*, *Thermus thermophilus*, and *E. coli*<sup>179</sup>. Up to date, no methylation of G18 in gram-positive bacterial tRNAs could be detected<sup>179</sup>. TrmK and TrmB are the only methyltransferases to have been identified in gram-positive bacteria to date (i.e. in *B. subtilis*). TrmK introduces a 1-methyl-adenosine at position A22 in tRNAs<sup>179</sup>. Methylation at position 18 by TrmH stabilises the structure of tRNA, and the immunomodulating effect is probably a positive side effect for the bacterium. However, all bacterial species tested except *E. coli* induced immune activation (Figure 5-4), indicating that immunosilencing modifications of tRNAs are not as prominent in all bacteria as in *E. coli*.

It was also demonstrated in the present study that the whole tRNA is not necessary for inhibition, but rather that a small 27-nucleotide long fragment of the tRNA containing the 2'-O-methylation at position 18 is sufficient (Fig. 5-3). This indicates that even fragments of tRNA, for example after cleavage by RNase, would be able to suppress immune activation in infections. The 2'-O-methylated fragment alone was not immunostimulatory; the same was true for the unmodified equivalent, which was used as control in the experiments. The total tRNA without any modifications was stimulatory, indicating that a minimum length was needed for stimulation. For inhibitory effects, smaller fragments are sufficient. By contrast, siRNAs with a length of 21–27 nucleotides could be recognised by TLR7<sup>118,207,208</sup>.

Therefore, sequence composition may play a role in recognition – as it has been shown that TLR7 has a preference for recognising GU-rich sequences<sup>31,32,115</sup>. Double-stranded structures could also play an important role in immune activation, as speculated by Eberle et al.<sup>187</sup>. After co-transfection with stimulatory RNA, IFN- $\alpha$  production was strongly reduced by inhibitory RNA and only minor effects were observed from control RNA.

### 6.1.2 The role of type-I interferons in bacterial infections

Most studies have analysed the non-stimulatory and inhibitory effects of modifications with respect to IFN- $\alpha$  stimulation via TLR7 in pDCs. TLR7 is known to be a receptor for viral and bacterial RNA<sup>31,32,200</sup>. It is expressed in pDCs and leads to production of IFN- $\alpha$  upon activation. The IFN- $\alpha$  released binds to type-I interferon receptor and induces expression of hundreds of interferon-stimulated genes (ISGs) in the surrounding cells and tissues. These cells create an antiviral state, resulting in suppression of viral infection and onset of adaptive immunity<sup>209,210</sup>. The key feature of the antiviral state is interaction of ISGs directly with the virus and the shutdown of the host protein translation machinery to prevent its use by the virus<sup>211</sup>.

Receptors such as TLR2 are capable of distinguishing between viral and bacterial proteins, which results in a different immune response<sup>212</sup>. To investigate this, Barbalat et al. used HEK293 cells stably expressing TLR2 and different knock-out mice to exclude the activation of different TLRs<sup>212</sup>. TLR2 can be activated by human cytomegalovirus<sup>213,214</sup>, herpes simplex virus<sup>215,216</sup>, hepatitis C virus<sup>217</sup>, measles virus<sup>218</sup>, and vaccinia virus<sup>219</sup>, leading to production of type-I IFNs. In contrast, recognition of bacterial cell wall components leads to induction of pro-inflammatory cytokines including TNF and IL12p40. By isolating different subtypes of bone marrow and spleen cells, Barbalat et al. identified different cells responsible for TNF and IL12p40 secretion and for IFN- $\alpha$  secretion in response to TLR2 activation<sup>212</sup>. Thus, the cell type is critical whether TLR2 can be activated by a viral or bacterial stimulus<sup>212</sup>. In contrast, activation of TLR7 results in production of type-I IFN in response to both viruses and bacteria. The production of type-I IFNs not only influences the antiviral immunity; type-I IFNs are also capable of influencing the antibacterial immune response<sup>220</sup>. It has been shown that low amounts of type-I IFNs prevent the intracellular replication of *Legionella pneumophila* in macrophages<sup>221</sup> and that type-I IFN treatment of mice that were intragastrically infected with *Salmonella typhimurium* reduced the lethality<sup>222</sup>. Thus, type-I IFNs appear to be protective against infections with gram-negative bacteria. By contrast,

experiments with mice deficient in type-I IFN production have shown an increased resistance to lethal effects of LPS, indicating a contribution of type-I IFNs to the lethal septic shock syndrome<sup>223</sup>. The underlying mechanism is elusive. For infections with gram-positive bacteria, treatment of mice with purified type-I IFN preparations or IFN-specific antibodies has indicated benefits of type-I IFNs for treatment of infections with *Streptococcus pneumoniae*<sup>224</sup>. The same positive effect has been observed for human and murine macrophages infected with *Bacillus anthracis*<sup>225</sup>. Upon treatment with type-I IFN, the cells were protected from the lethal effects of the bacterium and the intracellular spore germination was reduced. On the other hand, intravenous or intraperitoneal infection of mice deficient in IRF3, or signalling through IFNAR, indicated a reduction in host resistance in primary *Listeria monocytogenes* infections by the type-I IFN response<sup>223</sup>. Thus, the type-I IFN response is not only important in viral infections but also plays a role in defence against bacterial infections.

### 6.1.3 2'O-methylation inhibits production of pro-inflammatory cytokines

Most studies have investigated the effect of RNA modifications on human pDCs in which IFN- $\alpha$  production is induced via TLR7. We found that 2'O-methylated RNA not only lacked and blocked IFN- $\alpha$  production, but also reduced the production of pro-inflammatory cytokines including IL12p40 and TNF (Figure 5-6). One other group observed an inhibition of IL6 production by double-stranded siRNAs in which one strand was 2'O-methylated and inhibited the stimulation by its unmodified counterpart<sup>206</sup>. Another group observed inhibition of bRNA-mediated IFN- $\alpha$  induction by 2'O-methylated siRNA, but not inhibition of induction of TNF<sup>200</sup>. The lack of effect on TNF production may have been due to possible contamination of bRNA preparations with cell wall components such as LPS. Unlike monocytes, pDCs do not express TLR4 and are therefore resistant to LPS contamination. As the bRNA-induced immunostimulation in that study was abolished upon treatment with RNase, possibly a synergism between cell wall components and RNA could have led to the strong TNF induction. The RNA preparations used here were isolated by a two-step procedure, first with TRIzol followed by isolation over a silica membrane. In the latter procedure, RNA mixed with chaotrope salts binds to the silica membrane. The bound RNA is washed and then eluted. As no cell wall components can bind, adequate washing results in pure RNA solutions.

In the work for this thesis, one 2'O-methylated site was found to be sufficient to inhibit the stimulation of a wide range of cytokines including TNF, IL12p40, and IL6. By contrast,

in another study, a minimum of three methylated sites in siRNA was found to be necessary for inhibition of TNF production, while one methylation in 21 nucleotides was sufficient to inhibit IFN- $\alpha$  production<sup>206</sup>. This effect might be due to sequence specificity of the methylated motifs (see below).

TLR7 was shown to be expressed mainly in pDCs secreting type-I IFNs and it is debated whether IL6 is also secreted by pDCs. Induction of TNF and IL12p40 production by bRNA probably has to take place in another cell type. Fractionation of PBMCs in CD14-positive monocytes and CD14-negative cells led to production of IFN- $\alpha$  only in the whole PBMC preparation and the CD14-negative fraction, while IL12p40, TNF, and IL6 were produced by the whole PBMC preparation and by the CD14-positive fraction (Figure 5-9). This indicated that monocytes were responsible for production of pro-inflammatory cytokines upon transfection with bRNA. It has already been shown that monocytes do not express TLR7, but rather high amounts of the RNA-sensing receptor TLR8. TLR8 is responsible for induction of pro-inflammatory cytokines in monocytes in response to viral RNA, with a preference for AU-rich sequences<sup>102,152</sup>. Jöckel et al. were also able to detect production of IL12p40 and TNF in response to tRNA preparations in enriched monocytes<sup>124</sup>. In addition, monocytes did not respond to imiquimod, a ligand specific for TLR7, indicating that no TLR7 is expressed in monocytes. Thus, it appears that not only TLR7, but also TLR8 can be stimulated by bRNA and that this activation would be prone to inhibition by 2'-O-methylated RNA. However, the sensing of bacterial RNA by human TLR8 in monocytes has not yet been proven, and it could be addressed using knock-down experiments.

Taken together, the data support the influence of 2'-O-methylation of RNA in suppression of immunostimulation induced by RNA. This suppression is especially important for self RNA, as self RNA should not activate the immune system. If the discrimination of self RNA and non-self RNA fails, self RNA can lead to immune activation - resulting in an autoimmune disease. The immune diseases that result from recognition of self RNA are mainly SLE and psoriasis<sup>149-154</sup>. In SLE, it has been shown that autoantibodies are produced<sup>162</sup>. Here, differences in RNA modification in patients and in healthy individuals may play an important role, as preliminary data suggest (unpublished data).

#### **6.1.4 The inhibitory effect of 2'O-methylated RNA is specific for stimulation with RNA**

As the inhibitory RNA appeared to block the stimulation of TLR7 and TLR8, the inhibitory effect might apply to other TLRs or small synthetic TLR7/8 ligands. Therefore the TLR ligands CpG, R848, and Pam<sub>3</sub>CSK<sub>4</sub> (ligands for TLR9, TLR7/8, and the dimer TLR1/2, respectively) were used in co-transfections with the inhibitory RNA preparation and the cytokine production was measured (Figure 5-10). Inhibitory RNA might block the entry of the stimulatory agents or activate an inhibitory receptor influencing the activation of other TLRs. However, no reduction in stimulation by the different TLR ligands was observed. This result indicated that the inhibitory effect was specific for stimulation with RNA.

Remarkably, we did not even observe an inhibition of the effect of R848, even though it activates both TLR7 and TLR8. This might be due to different binding sites for R848 and RNA at the receptor, as reported by Colak et al.<sup>130</sup>. R848 is often described as a guanosine analogue, but the binding to its receptor clearly differs from that of the natural ligand. This might be due to the chemical composition or to the size. Colak et al. recently found that R848 induced different phosphorylation and transcriptional events after activating TLR8 compared to RNA. In addition, TLR7 and TLR8 recognise RNA and R848 by overlapping and non-overlapping recognition sites, and discriminate between both<sup>130</sup>. To investigate this, TLR7 and TLR8 were mutated at different positions of the ectodomains and activation by the two ligands was observed. It could be shown in this thesis that the inhibitory RNA binds directly to the receptor. R848 might still bind to TLR7 and TLR8 through different binding sites, resulting in an activation of the receptor even in presence of the inhibitory RNA.

#### **6.1.5 No other immunosilent RNA preparations were identified**

*E. coli* tRNA and *T. thermophilus* tRNA preparations have been shown to be non-stimulatory, resulting from 2'O-methylations incorporated in RNA nucleosides<sup>124</sup>. This feature could influence the recognition of the whole bacterium by the immune system. It could therefore be used as an immune evasion mechanism<sup>124</sup>. Identification of other non-stimulatory RNA preparations could help our understanding of the beneficial or harmful properties of some bacteria.

Thus, different tRNA preparations were tested for their stimulatory capacity. In the work for this thesis, all the tRNA preparations tested induced IFN- $\alpha$ , IL12p40, and TNF production (Figure 5-5 and 5-8). The general ability of tRNA preparations to induce IFN- $\alpha$  in PBMCs was also described by Jöckel et al.<sup>124</sup>. They also reported that different levels of cytokines

were induced by tRNA preparations from different species. In addition, they showed that tRNA preparations from *E. coli Nissle* and *T. thermophilus* lacked immune stimulation regarding IFN- $\alpha$  production<sup>124</sup>, which might be exceptions to the rule. Both tRNA preparations were shown to inhibit the production of IFN- $\alpha$  induced by other immunostimulatory tRNAs, in a dominant fashion<sup>124</sup>. Yet surprisingly, *T. thermophilus* tRNA has been found to induce production of IL6 in human PBMCs<sup>124</sup>. As *T. thermophilus* is a gram-negative bacterium, this might be due to LPS contamination activating TLR4. Other studies have also identified single tRNAs from *Saccharomyces cerevisiae* as being immunosilent. However, in contrast to the immunosilent tRNA preparations identified, where 2'-O-methylation at position G18 could play an important role, the immunosilencing by mammalian tRNA<sup>Phe</sup> and tRNA<sup>Lys</sup><sub>3</sub> is independent of Gm18<sup>123,179</sup>. This indicates that further immunosilencing modifications are used in native tRNAs, and are important. The exact modifications responsible have not been identified so far.

As *E. coli Nissle* is known as a bacterium used for probiotics, Jöckel et al. discussed that the inhibitory effect of the tRNAs may support this effect<sup>124</sup>. Probiotics should provide health benefits when being consumed. They modify the flora of humans and replace harmful bacteria with useful ones. *E. coli Nissle* is also used as therapeutic to treat chronic disease such as inflammatory bowel disease (IBD). *Bifidobacterium bifidum* is also known for probiotics and it can be used to rebuild a healthy flora in the colon. In the present study, the tRNA preparation of *Bifidobacterium bifidum* induced IFN- $\alpha$  production. The effect observed by Jöckel is therefore not true for all probiotic bacteria.

The stimulatory effects of all the RNA preparations tested could be inhibited by co-transfection with inhibitory 2'-O-methylated RNA, indicating that the inhibition is not specific for tRNA or RNA from a defined species. In addition, the group of Bauer showed that stimulation by RNA from influenza A virus - which triggers TLR7 activation - could also be inhibited by 2'-O-methylated RNA<sup>124</sup>.

All tRNA preparations tested induced cytokine production, but at different levels, ranging from 1–2.5 ng/ml IFN- $\alpha$ , 300–1,000 pg/ml IL12p40, and 200–800 pg/ml TNF when excluding the outlier *Bacillus subtilis*. To screen the low-stimulatory preparations for non-stimulatory or even inhibitory single tRNAs, fractionation of the preparations could be done. This could, for example, be performed by size-exclusion chromatography. The resulting fractions could then be tested for their stimulatory potential, and non-stimulatory fractions could be screened for their exact content, especially for modifications.

## **6.2 The mechanism of inhibition indicates direct binding of 2'O-methylated RNA to TLR7**

To investigate the mechanism of inhibition, we analysed the downstream signalling of TLR7 and TLR8 upon RNA stimulation and the expression of mRNA for different cytokines. We found that the inhibitory effect of the 2'O-methylated RNA could be observed at the level of mRNA expression as well as at the level of MAPK and NFκB activation (Figures 5-13 and 5-14). The decrease in activation was observed at early time points, while the control RNA failed to inhibit the activation, indicating that the 2'O-methylated RNA interacted directly with the receptor. The results also indicated that R848 induced activation within the first 60 min, whereas stimulation with RNA was not apparent before 120 min. The cellular uptake of the small molecule R848 differs from that of the encapsulated RNA, resulting in different activation times (Figure 5-13). In addition, the observation that co-transfection with stimulatory and inhibitory RNA was necessary for proper inhibition indicated direct competition at the receptor - or a need for spatial restriction of an inhibitory receptor to the stimulated one - to inhibit the activation directly (Figure 5-15). Sequential uptake of RNA was observed in live-cell imaging of fluorescent-labelled RNA at later time points (Figure 5-19). The fluorescence was first located in dot-like structures, before showing release into the cytoplasm. The signal vanished after a while, before dot-like structures could again be seen. This indicated that there was repeated cellular uptake of RNA-containing liposomes and release of RNA into the cytoplasm. This could explain the differences in stimulation observed for time-delayed addition of stimulatory and inhibitory RNA. A 50% inhibition for transfection with the inhibitory RNA 3 h after the stimulatory one was also observed by Hamm et al. for production of IFN-α and TNF in human PBMCs<sup>206</sup>.

### **6.2.1 2'O-methylated RNA probably competes with stimulatory RNA for receptor binding**

Co-immunoprecipitation experiments with 2'O-methylated or unmethylated RNA and TLR7 illustrated that the inhibitory RNA probably bound directly to TLR7. We showed that 16S rRNA bound to TLR7 (Figure 5-16). 16S rRNA could be displaced by adding inhibitory RNA. To determine the amount of 16S rRNA bound to TLR7, the RNA was pulled down with TLR7 and isolated. The resulting RNA was reverse transcribed to DNA for analysis by qPCR. Some 16S rDNA strands were also detected in the negative sample, where no RNA was used for transfection. This might be due to known contamination of the reagents used for



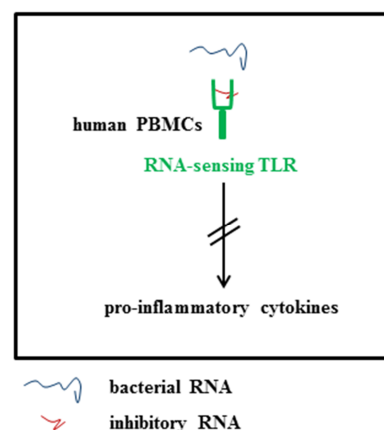
isolation and synthesis. Most of the enzymes used are produced by recombinant techniques in bacteria, so the enzymes isolated may have contained bacterial nucleic acids recognised by the primers used, which detected conserved regions of the 16S rDNA sequence<sup>226</sup>.

In a second approach, TLR7 was detected after pull-down with biotinylated RNA (Figure 5-17). In this way, it could be shown that TLR7 bound to the unmodified RNA. On the western blot, TLR7 co-immunoprecipitated with RNA was detected as two bands that migrated faster than TLR7 alone. This observation might suggest that there was cleavage of the receptor. In 2011, Ewald et al reported that TLR9 was cleaved during processing in endosomes<sup>227</sup>. The same was found for TLR3 and TLR7<sup>227</sup>. TLR7 was cleaved twice on the way from the ER to the endosome. This might explain our own observations. As the HA tag was detected by our antibody, only the parts of TLR7 that were still attached to the tag would be detected. As two bands were observed, the upper band could be the cleavage product of the first cutting, while the lower band may have been the result of two cleavages. The TLR7 pulled down with anti-HA beads binds to the beads after cell lysis. Thus, it could also be located in the ER before lysis where it is still uncleaved.

To demonstrate that the inhibitory RNA not only replaced the stimulatory RNA from the receptor but bound directly to the receptor, biotinylated tRNA with and without Gm18 was used for co-immunoprecipitation with TLR7 (Figure 5-18). Both tRNAs were able to pull down TLR7, indicating that both RNAs bound directly to the receptor. The amount of the co-immunoprecipitated protein was similar in both approaches. Again, the displacement of the unmodified tRNA through the methylated tRNA was investigated. Biotinylated tRNA carrying or not carrying a 2'O-methylation at G18 was used for co-transfection with its unbiotinylated counterpart. The results clearly indicated that the methylated RNA could displace the unmodified one from the receptor, but not vice versa. The biotinylated, unmodified RNA was unable to pull down TLR7 when 2'O-methylated RNA was present. On the other hand, biotinylated 2'O-methylated RNA precipitated the same amount of TLR7 in the presence and absence of unmodified RNA. Together, these data indicate a stronger binding affinity of 2'O-methylated than of unmethylated tRNA for TLR7, as the 2'O-methylated tRNA was able to displace the unmodified tRNA. Therefore, we believe that the 2'O-methylated RNA binds directly to the receptor, blocks it, and hinders activation by stimulatory RNA as shown in Figure 6-1. The assay used is limited, due to the receptor being located in the endosomes, RNA reaching the endosomes, and the sensitivity of the analysis.

The binding of 2'-O-methylated RNA to TLR7 was also investigated by Hamm et al. They used 2'-O-methylated and unmodified siRNA with a human TLR7 protein in an amplified luminescent proximity homogenous binding assay (AlphaScreen™). They observed a very efficient binding of 2'-O-methylated siRNA to TLR7, while the unmodified counterpart bound more weakly<sup>206</sup>. The binding was observed separately for the 2'-O-methylated and unmodified siRNAs, and no direct competition of the siRNAs at TLR7 was studied. Other studies have investigated the stoichiometry and structural constitution of the RIG-I-dsRNA complex by gel filtration and scanning force microscopy<sup>228</sup>. Thus the ligand-receptor complex was filtrated and the different fractions were analysed with photometry. By shifting the peaks for protein or nucleic acid detection, the binding of the two components could be shown. By using scanning force microscopy, a model of the complex surface was produced, enabling conclusions about the ratio of ligand to receptor in the complex.

The efficiency of binding of the inhibitory RNA to TLR7 and TLR8 was slightly different, as titration studies indicated (Fig.5-11). PBMCs were stimulated with bRNA involving co-transfection with different amounts of inhibitory RNA. Production of IFN- $\alpha$ , TNF, and IL12p40 was measured and the IC<sub>50</sub> calculated. The IC<sub>50</sub> to inhibit 1  $\mu$ g/ml stimulatory RNA was calculated as 0.06  $\mu$ g/ml at TLR7, while the IC<sub>50</sub> at TLR8 was calculated as 0.01–0.02  $\mu$ g/ml, indicating that the inhibitory RNA might have a stronger affinity for TLR8 than for TLR7. The concentration of the inhibitory RNA at IC<sub>50</sub> corresponds to 1/50 to 1/100 of the stimulatory concentration for TLR8. Other groups have also analysed the inhibition; they came up with an IC<sub>50</sub> of 15–5 nM for all cytokines and receptors, reflecting 1/65 to 1/200 of stimulatory RNA concentration<sup>206</sup>, which is therefore comparable to our own data. The IC<sub>50</sub> of the unmethylated control RNA was also calculated in our study. It was around 10 times higher, indicating that the 2'-O-methylated RNA had a higher affinity for the receptor than the unmethylated one.



**Fig. 6-1: Overview of the proposed mechanism of immunoinhibition.** The inhibitory RNA binds to the receptor in human PBMCs, avoids activation, and blocks it from activation by stimulatory RNA.

## 6.2.2 2'-O-methylated and unmodified RNA co-localise in liposomes and endosomes

The efficiency of the co-transfection was assessed in microscopy experiments. Thus, methylated and unmodified RNA labelled with different fluorescent dyes (ATTO488 and ATTO590) were used to co-transfect murine DCs and live-cell analysis was performed for 6 h. The two fluorescent-labelled RNAs were mixed before packaging with lipofectamine. Co-localisation was observed in dot-like structures outside and inside the cells, indicating location in liposomes and endosomes, respectively. Thus, both RNAs appeared to reach the same endosomes and could therefore compete for receptor binding. The location in endosomes was not shown unequivocally.

Surprisingly, a release of the fluorescence in the cytoplasm was observed, resulting in distinct localisation of the different dyes. While ATTO488-labelled RNA was located mainly in the cytoplasm, ATTO590-labelled RNA migrated to the nucleus. This localisation was dye-dependent and independent of the modification, as experiments with switched dyes resulted in the same distribution of fluorescence. Migration of transfected nucleic acids into the nucleus was also observed by Fisher et al.<sup>229</sup> and Leonetti et al.<sup>230</sup> in their studies using microinjection experiments in non-immune cells. A migration not only of DNA, but also of RNA, as observed here, might be possible due to passive diffusion through the nuclear membrane. Oligonucleotides of up to 100 bp can diffuse passively through the nuclear core complex<sup>231</sup>; for molecules, the diffusion limit has been shown to be 40 kDa<sup>232</sup>. This diffusion is independent of concentration, temperature, and ATP levels<sup>231</sup>. The green fluorescent signal from ATTO488-labelled RNA was distributed in the cytoplasm. This might have been due to the size of the dye. ATTO488 is a larger molecule than ATTO590, and coupled to the RNA this might result in a molecule larger than 40 kDa - the size limiting diffusion into the nucleus. Both fluorescent dyes are positively charged, but show differences in molecular structure; thus, dye-specific differences could have influenced the distribution. However, both fluorescence signals vanished after a while. This might have been due to degradation of the RNA and the fluorescent dye attached. The half-life of free DNA inside a cell averages 1–2 h, while DNA packed in lipoplexes remains stable for a longer time<sup>233,234</sup>. This encapsulation might explain that after degradation of nucleic acids in the cell, further lipoplexes could fuse with the cell and intact RNA would again be released in the cytoplasm. It has been shown in HeLa cells that all microinjected mRNA ends up in the nucleus, irrespective of the compartment into which the mRNA was injected. In contrast, the microinjection of tRNA strands resulted in

cytoplasmic localisation. This was observed for tRNAs injected into the cytoplasm as well as into the nucleus. The export of all tRNAs in the cytoplasm was attributed to the tRNA export system, which also exported unnatural tRNAs when they were introduced in high amounts<sup>235</sup>.

In the present work, we observed a periodic release of the RNA into the cytosol from dot-like structures, followed by complete disappearance of any signal, after which dot-like structures could again be observed. The RNA was packed in liposomes for transfection, visible as dot-like structures around the cells. After uptake into the cells, dot-like structures could again be observed, indicating their localisation in endosomes and endolysosomes.

To visualise the distribution of RNA, murine DCs were used in our study, as they are immune cells with a relatively large cytoplasm. However, as shown later, murine cells were not consistently inhibited by the RNA modification used. This might be due to the importance of TLR13 in murine immune cells. TLR13 has been shown to sense one specific sequence, which is conserved in 23S rRNA of bacteria<sup>36</sup>. Incorporation of N-6 methylation at a distinct position inside this sequence mediated an immunosilencing effect. The RNA sequences used for microscopic analysis were stimulatory in human cells. Therefore, live-cell experiments with human PBMCs would be a more appropriate model to study RNA distribution. PBMCs are a mixture of different cells, and only a subset of them is stimulated by the RNA. To obtain a more unique cell population, distinct populations should be isolated beforehand, such as monocytes or pDCs. However, both cells are known to have a large nucleus surrounded by only a small cytoplasm. Inside these cells, the distribution of fluorescent-labelled RNA inside the cytoplasm would be difficult to observe. Other human cells with a large cytoplasm should be used, but if they are not specialised immune cells the distribution might differ because no recognition via TLRs would take place in these cells. Alternatively, the imaging could be done with RNA containing an N-6 methylation, using murine cells to observe differences for the murine system.

### **6.3 Inhibitory RNA in the murine system**

As the blocking of cytokine production by co-transfection with stimulatory RNA and the 2'-O-methylated inhibitory RNA showed clear results in the human system, and as TLR7 in the murine system is known to have similar specificities to the human one, the same effect was studied in murine immune cells. The results were not as clear as in the human cells. In many cases the blocking of cytokine secretion by 2'-O-methylated RNA could be observed, but not in all experiments and it was not as prominent as in the human system. In particular,

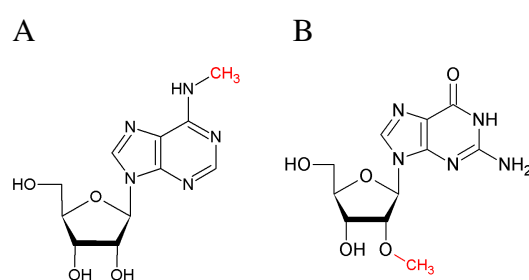
differences were observed between different batches of GM-CSF-containing conditioned medium generated by the plasmacytoma X63 cell line, which was used for generation of bone marrow-derived DCs. In other studies, using murine FLT3L-DCs, inhibition of IFN- $\alpha$  and IL6 expression by 2'-O-methylated RNA was detected<sup>206</sup>. The FLT3L-DCs used are the murine-equivalent cells to human pDCs and they are known to express TLR7, while in our study DCs generated with GM-CSF were used (cDCs). The cells generated could have differed depending on different batches of the differentiation medium, as we could not ensure that an identical amount of GM-CSF was present. cDCs express TLR13 and produce pro-inflammatory cytokines including IL12p40 and TNF. pDCs express TLR7 and are the main producer of type-I IFN.

We checked the TLR levels of cells generated with different batches of GM-CSF. While the levels of TLR7 and TLR13 showed only small differences, TLR3 was expressed at high levels in one sample, and almost no TLR3 expression could be detected in the other one. While inhibition was observed in the cells with less TLR3, no inhibition was detected in cells expressing high levels of TLR3. TLR3 is

known to detect double-stranded RNA. Thus, it is possible that the RNA used activated TLR3 under certain circumstances or that the 2'-O-methylated RNA bound to TLR3 with high affinity and could therefore not block other receptors. This would then indicate that TLR3 could not be inhibited by methylated RNA. Stimulation of the cells with polyI:C, a ligand for TLR3, was not tested.

Furthermore, the ratio of expression of TLR7 and TLR13 could play a role. While TLR7 senses ssRNA, TLR13 recognises one specific sequence. This sequence is conserved in 23S rRNA of bacteria and is therefore present in the bRNA preparation that we used. The sequence stimulating TLR13 can be silenced by incorporation of an N-6 methylation at a defined adenosine<sup>36</sup>; the methylated base is shown in Fig. 6-1A. In the present study, we used 2'-O-methylation of guanosine to inhibit immune activation (shown in Fig. 6-2B). Different modifications are probably responsible for inhibition of the different receptors, namely N-6 methylation for TLR13 and 2'-O-methylation for TLR7.

Also, the role of cytosolic RNA receptors was not investigated and they could also play a role in the stimulation by the RNA introduced by transfection. If this is true, it should be



**Fig. 6-2: Structure and location of different RNA modifications.** (A) N-6 methylation of adenosine. (B) 2'-O-methylation of the ribose in guanosine<sup>179</sup>.

determined whether their activation can be inhibited by any modification. As RNA is introduced with cationic lipoplexes into the cells, it cannot be checked that the RNA is specifically delivered into endosomes. As observed by microscopy, RNA was released into the cytoplasm where cytosolic receptors could be activated. But, stimulation of cytosolic receptors mainly results in production of type-I IFNs. As the activation of TNF and IL12p40 production was measured, an activation of TLRs is more likely as they are known to induce pro-inflammatory cytokines including TNF and IL12p40.

To investigate this issue further, experiments could be performed by co-transfection with stimulatory RNA together with 2'-O-methylated and N-6 methylated RNA to inhibit TLR7 and TLR13 in murine cells. As TLR13 recognises only a specific sequence in 23S rRNA, RNA preparations lacking the 23S rRNA could be used for stimulation.

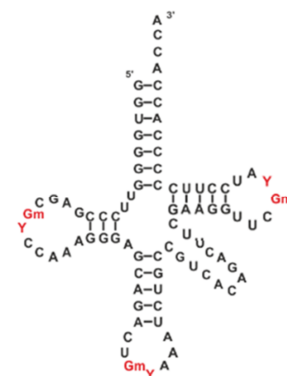
#### **6.4 Identification of an immunosilencing modification motif**

In the last part of this work, a sequence motif responsible for the immunosilencing effect was studied. We found that the three-dimensional structure stabilised by the 2'-O-methylation at guanosine 18 probably had no influence on the immunosilencing capacity of the tRNA (Figure 5-24). This may be because RNA is recognised in the endosomes inside the cell. Endosomes fuse with lysosomes, which are specialised in destroying invading pathogens. The low pH inside the vesicular bodies<sup>236</sup> may lead to destruction of the hydrogen bonds stabilising the three-dimensional structure of the tRNA. Thus, the three-dimensional structure might be destroyed in the vesicle where it meets TLR7.

Permutation studies of the methylated nucleoside indicated that all nucleosides except cytidine led to immunosilencing (Figure 5-25 - 5-27). For the base following the methylation, a purine (guanine or adenine) was identified to be important for the immunosilencing effect. This raised the question of whether the methylation at position 18 formed an inhibitory motif or whether it destroyed a stimulatory motif. TLR7 was shown to recognise GU-rich sequences and TLR8 has been found to prefer AU-rich sequences. According to our data, incorporation of a 2'-O-methylation in AU or GU motifs resulted in a stimulation. This indicates that the immunosilencing effect is not due to disturbance of a recognised sequence by incorporation of a 2'-O-methylation.

Comparison with other studies that used 2'-O-methylated RNA for inhibition confirms the relevance of the sequence motif identified. Hamm et al. analysed siRNAs with different amounts of 2'-O-methylation for their immunosilencing and immunoinhibitory capacity when annealed to an unmodified strand. They transfected PBMCs with the siRNAs and analysed the supernatants for IFN- $\alpha$  and TNF production. The study indicated that one 2'-O-methylated nucleoside would be sufficient for immunosilencing of IFN- $\alpha$  and TNF production when transfecting with the 2'-O-methylated siRNA strand alone. After transfection with the annealed siRNA (the 2'-O-methylated strand plus the unmodified strand), three 2'-O-methylated nucleosides were found to be necessary for inhibition of TNF production<sup>206</sup>. A closer look at the sequence of the 2'-O-methylated siRNA used indicated that the first methylation resulted in an AmC motif. A cytidine located at position +1 upstream of the methylation influenced inhibition negatively in our experiments. The second methylation incorporated by Hamm et al. resulted in the motif CmC. Based on the data presented in our study, incorporation of CmC within siRNA would have no inhibitory activity. To introduce the third methylation, the second one was shifted to a CmG motif. The third methylation resulted in an AmA. This third modification, according to our data, would be the most efficient one for immunosilencing and inhibition. So it was not surprising that incorporation of three 2'-O-methylations was necessary to obtain a dominant immunoinhibition for the cytokines tested. Incorporation of the third methylation alone should have the same effect and should silence the stimulation. Our data indicate that 2'-O-methylation alone is not sufficient but that the sequence motif also plays an important role. This could be used for RNA-based therapeutics, in which one 2'-O-methylation at the correct position could be sufficient to avoid stimulatory side effects and a modification of the whole RNA would not be necessary. This could minimise unwanted interactions between incorporated modifications and could make generation and synthesis of siRNAs easier. siRNAs as drugs against different cancers and viral infections are being tested in different clinical trials<sup>237</sup>. It was also speculated by Eberle et al. that by incorporation of the methyl group, the binding of hydrogen bonds by the 2'-OH group is abolished. The methylation further reduces the chance that the 2'-OH group can act as H-bond acceptor<sup>187</sup>.

Furthermore, the 2'-O-methylated motif was shifted to two other positions inside the tRNA as indicated in Figure 6-3 (Figure 5-28 and 5-29). At all positions similar motif specificities were observed.



**Fig. 6-3: Sequence of *E.coli* tRNA<sup>Tyr</sup> with the methylation sites indicated in red.**

This contradicts the hypothesis of destroying a stimulatory motif by 2'O-methylation. Three different positions in the tRNA were 2'O-methylated leading to an immunosilent RNA, indicating that all three positions had to be stimulatory before, resulting in a minimum of three stimulatory motifs. For disturbing all stimulatory motifs a minimum of three 2'O-methylations have to be incorporated. However, in each tRNA version tested, only one motif was 2'O-methylated. The other two motifs would remain stimulatory. In this case, the stimulation of the tRNA would potentially be reduced compared to the unmodified one, but no complete silencing would be expected. Furthermore, silencing of a stimulatory motif would not inhibit the stimulation by other RNA strands. Therefore, generation of an inhibitory motif by 2'O-methylation is more likely.



## 6.5 Outlook

In the present work, we analysed inhibition of TLR7 and TLR8 signalling by 2'O-methylated RNA. The involvement of TLR8 was not formally proven in this study. For verifying this, knock-down experiments in primary cells could be performed and TLR8 could be overexpressed in HEK cells and checked for NFκB activation. Preliminary data indicate that there is direct binding of the 2'O-methylated RNA to TLR7. This should be verified by other methods using the isolated receptor. AlphaScreen™ analysis or gel filtration could be used. TLR8 should also be examined in the same way.

The dinucleotide motif identified to be responsible for immunosilencing of *E. coli* tRNA<sup>Tyr</sup> was tested at different positions inside the tRNA. It could be incorporated into tRNAs of other bacterial species to determine whether the motif is generally responsible for immunosilencing or whether the effect observed is specific for *E. coli* tRNA. Also, the amount of 2'O-methylation at position G18 in whole tRNA preparations of different species - bacterial, fungal, and mammalian - could be analysed and correlated with their stimulatory capacity. This would indicate the importance of this specific modification in discriminating between self and non-self structures.

tRNAs of the fungus *Saccharomyces cerevisiae* (tRNA<sup>Phe</sup>, tRNA<sup>Lys3</sup>) were identified to be immunosilent independently of Gm18. Here, the modification responsible could be identified and compared to Gm18.

To understand the relevance of RNA modifications in autoimmune disease such as SLE, RNAs released by apoptotic cells from SLE patients could be analysed for the amount and kind of modifications, and compared to those from healthy individuals. In SLE patients, self RNA activates immune receptors resulting in the formation of autoantibodies. Preliminary analyses in this field indicate that there are differences in the frequency of some modifications.

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## 8 PUBLICATIONS AND PRESENTATIONS

### 8.1 Publications

Kaiser S<sup>#</sup>, **Rimbach K**<sup>#</sup>, Eigenbrod T, Dalpke AH, and Helm M.

A modified dinucleotide motif specifies tRNA recognition by TLR7

RNA, accepted

<sup>#</sup> equal contribution

Bretz NP, Ridinger J, Rupp AK, **Rimbach K**, Keller S, Rupp C, Marmé F, Umansky L, Umansky V, Eigenbrod T, Sammar M, Altevogt P.

Body fluid exosomes promote secretion of inflammatory cytokines in monocytic cells via Toll-like receptor signaling.

J Biol Chem. 2013 Dec 20;288(51):36691-702.

Gehrig S, Eberle ME, Botschen F, **Rimbach K**, Eberle F, Eigenbrod T, Kaiser S, Holmes WM, Erdmann VA, Sprinzl M, Bec G, Keith G, Dalpke AH, Helm M.

Identification of modifications in microbial, native tRNA that suppress immunostimulatory activity.

J Exp Med. 2012 Feb 13;209(2):225-33

### 8.2 Presentations

Poster: Inhibition of innate immune activation by bacterial RNA modifications

Katharina Rimbach, Alexander Dalpke, Tatjana Eigenbrod

24th European Congress for Clinical Microbiology and Infectious Disease, Barcelona, Spain,

May 2014

Talk: Mechanisms of action of inhibitory bacterial RNA modifications

Katharina Rimbach, Tatjana Eigenbrod, Alexander Dalpke

18th Minisymposium „Infektion und Immunabwehr“, Rothenfels, March 2014

Poster: Mechanisms of immune inhibition by modified bacterial RNA

Katharina Rimbach, Tatjana Eigenbrod, Alexander Dalpke

43rd Annual Meeting German Society for Immunology, Mainz, September 2013

Talk: Inhibitory bacterial RNA modifications as immune escape mechanism

Katharina Rimbach, Tatjana Eigenbrod, Alexander Dalpke

17th Minisymposium „Infektion und Immunabwehr“, Rothenfels, March 2013

Talk: FRET-based real-time analysis of siRNA integrity and distribution in living cells

Katharina Rimbach, Markus Hirsch, Alexander Dalpke and Mark Helm

PhD/Postdoc Colloquium of the research programs ‘Methoden für die Lebenswissenschaften’,  
Bad Herrenalb, April 2012

Poster: Observation of the 2’O-methyl modification on RNA which suppress  
immunostimulation

Katharina Rimbach, Alexander Kazakov, Tatjana Eigenbrod, Alexander Dalpke

8th Spring School on Immunology, Ettal, March 2012

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