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Original article

Delivery with polycations extends the immunostimulant Ribomunyl® into a potent antiviral Toll-like receptor 7/8 agonist

Stephan Herberhold[1,2†], Christoph Coch[1†], Thomas Zillinger[1], Benjamin Hommertgen[1], Nicolas Busch[1], Christine Schuberth[1], Evelyn Hartmann[1,2], Vera Wimmenauer[1], Cristina Amparo Hagmann[1], Bastian Lüdenbach[1], Martin Schlee[1], Friedrich Bootz[2], Gunther Hartmann[1], Winfried Barchet[1*]*

[1]Department of Clinical Chemistry and Clinical Pharmacology, University Hospital of Bonn, Bonn, Germany
[2]Department of Oto-Rhino-Laryngology, University Hospital of Bonn, Bonn, Germany

*Corresponding author e-mails: Winfried.Barchet@ukb.uni-bonn.de; ccoch@uni-bonn.de
†These authors made an equal contribution to this work

Background: Upper respiratory tract infection is a frequent cause of morbidity worldwide. Although the course of infection is usually mild, it is responsible for enormous social and economic costs. Immunostimulation with bacterial extracts consisting of ribosomal RNA and proteoglycans, such as Ribomunyl®, was introduced into the clinic in the 1980s as a new treatment concept, but did not achieve widespread application. Ribomunyl® has been proposed to activate innate immunity, but the contribution of its RNA content as well as its antiviral potential has not been studied.

Methods: Peripheral blood mononuclear cells from healthy donors and immune cells from adenoids were incubated with Ribomunyl® either by itself or formulated in a complex with cationic polypeptides such as poly-L-arginine or protamine, and induction of cytokines was quantified by ELISA.

Results: Ribomunyl® in complex with either poly-L-arginine or protamine, but not on its own, was able to strongly induce interferon-α ($P<0.01$) and interleukin-12 ($P<0.01$) in peripheral blood mononuclear cells, whereas induced tumour necrosis factor-α and interleukin-6 levels were independent of the formulation. Comparable results were obtained in immune cells from adenoids, suggesting efficacy also in virus-affected tissue. Cell sorting, RNase digests and selective receptor expression show that the RNA in Ribomunyl® acts as an agonist of Toll-like receptor (TLR)7 and TLR8.

Conclusions: Ribomunyl® is, in principle, able to potently induce antiviral interferon-α and interleukin-12 via TLR7 and TLR8, respectively, but only when formulated in a complex with cationic polypeptides.

Introduction

Upper respiratory tract infection (URTI) is a collective term that summarizes the common and frequently occurring bacterial and viral infections of the nose, sinuses, pharynx and larynx [1,2]. Because of its high incidence, URTI impairs the economy – incurring an enormous number of sick days – and is therefore of significant epidemiological importance [2]. Although mostly banal and non-life-threatening, URTI can trigger exacerbations of chronic lung disease with subsequent reduced quality of life and overall prognosis [3–6]. URTI can occur as polymicrobial disease; for example, a bacterial superinfection might complicate a primary viral infection [7–9]. Bacterial infections are routinely treated with antibiotics even though the efficacy in these diseases is called into question [10–12]. No specific antiviral therapy is available for viral URTI, and thus therapy remains limited to the treatment of symptoms. To develop a treatment option for URTI by activating the immune system, viral or bacterial lysates such as Ribomunyl® were introduced in the 1980s. According to the German regulatory authorities, Ribomunyl® is approved for marketing in over 100 countries worldwide as an oral drug. Published clinical trials and meta-analyses show some benefit of Ribomunyl® treatment in different clinically defined URTI in children and adults (for example, pharyngotonsillitis, otitis media and infectious rhinitis [13–17]), but this has not led to its routine use in the clinic. Ribomunyl® contains ribosomes from *Klebsiella*

pneumoniae, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Streptococcus pyogenes* group A, as well as proteoglycans from *K. pneumoniae*.

The innate immune system is equipped with germline-encoded pattern recognition receptors such as the family of the Toll-like receptors (TLRs) that are able to detect conserved pathogen-associated molecular patterns contained in viruses and bacteria leading to immune activation and subsequent eradication of the pathogen [18–23]. As a mechanism of action, it has been shown that the proteoglycans contained in Ribomunyl® can act as a TLR2 agonist [24–26]. Yet, even though RNA is the main component of Ribomunyl®, it has so far not been evaluated whether, and in what formulation, Ribomunyl® is able to activate also TLR7 and TLR8 in the endosome of immune cell subsets that function to detect the presence of pathogen RNA [19,27].

We found that Ribomunyl® on its own induced proinflammatory but not antiviral cytokines. By contrast, when delivered to the endosomal compartment in a complex with polycations such as protamine, the ribosomal RNA contained in Ribomunyl® induced high antiviral cytokines, not only in peripheral blood mononuclear cells (PBMC), but also in immune cells from virally colonized adenoid tissue [28] obtained from patients undergoing adenoidectomy because of recurrent infections. By RNase digest and receptor studies we show that the RNA induces cytokines by activating TLR7 and TLR8.

Methods

Immunostimulatory agents

Ribomunyl® (0.75 mg ribosomes from *K. pneumoniae*, *S. pneumoniae*, *H. influenzae*, *S. pyogenes* group A and 1.125 mg proteoglycans from *K. pneumoniae* per tablet) was kindly provided by Pierre Fabre (Castres, France). RNA was isolated from Ribomunyl® by phenol–chloroform extraction. The TLR agonists used were CpG ODN 2216 (TLR9 [29]; Metabion, Martinsried, Germany), Oligoribonucleotide (ORN) 9.2s RNA (TLR7/8 [30]; Biomers, Ulm, Germany), R848 (TLR7/8; Alexis, Lörrach, Germany), Cl-075 (TLR8) and Cl-087 (TLR7; Invivogen, Toulouse, France). PolyA (Sigma–Aldrich, St Louis, MO, USA) was used as the non-stimulatory RNA.

Preparation, isolation and culture of cells

Nasopharyngeal tonsils (adenoids) were obtained from patients undergoing elective adenoidectomy at the Department of Otorhinolaryngology, University of Bonn, Bonn, Germany (approved by the ethics committee, University of Bonn). Adenoids were minced in RPMI 1640 medium (Sigma–Aldrich), digested for 10 min with collagenase IV (Sigma–Aldrich) and passed through 70 μm nylon cell strainers. Cell suspension from adenoids and PBMC isolated from buffy coats of healthy blood donors were prepared by density gradient separation (Biochrom, Berlin, Germany). Plasmacytoid dendritic cells (PDC) were enriched to >95% purity, or depleted from cell suspensions by magnetic-activated cell sorting using the BCDA-4 dendritic cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC and immune cells from adenoid tissues were cultured at a density of 400,000 cells/well, and PDC at 40,000 cells/well in 96-well flat-bottom plates in 200 μl RPMI 1640 (Biochrom), 10% (v/v) fetal calf serum (Invitrogen, Karlsruhe, Germany), 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich). To enhance viability, PDC were treated with 5 ng/ml interleukin (IL)-3 (PeproTech, London, UK) [31].

Formulation of complexes of RNA with poly-L-arginine or protamine

Samples of 200 ng RNA or Ribomunyl® were diluted in 15 μl phosphate-buffered saline before 200 ng poly-L-arginine (pArg; Sigma–Aldrich) were added, incubated for 10 min and added to cells at a final concentration of 1 μg/ml. Alternatively 2 μg RNA or Ribomunyl® were mixed with 1.43 μg protamine (50 mg/ml Protamin Valeant®; MEDA Pharma, Cologne, Germany) and 0.75 μl NaCl (10%) and diluted with water to 8 μl. Then 5 μl of this mixture was added per well. For dose tritration, Ribomunyl® and pArg/protamine were changed proportionally and the volume kept constant.

TLR7/8 and TLR2 Luc assay

TLR-expressing and non-expressing HEK293 cells (Invivogen) transfected with pNF-κB-Gluc reporter plasmid were plated at 20,000 cells/well in 96-well plates, and after 12 h cells were stimulated with 50 ng/ml Pam2CSK4 (Invivogen), 1 μM small-molecule ligands or 25 μg/ml RNA in complex with polycationic peptides. Supernatants were collected after 16 h and Gaussia luciferase activity was determined using coelenterazine (Sigma–Aldrich).

ELISA

Cytokines were measured in cell culture supernatants according to the manufacturer's recommendations by the interferon (IFN)-α module set (Bender MedSystems, Graz, Austria), BD OptEIA TNF ELISA, BD OptEIA IL-12 (p70) or BD OptEIA IL-6 (BD Biosciences, Heidelberg, Germany).

Statistical analysis

Bars in the figures indicate the mean and standard deviation for the representative experiment shown, or for those that show means of two or three experiments

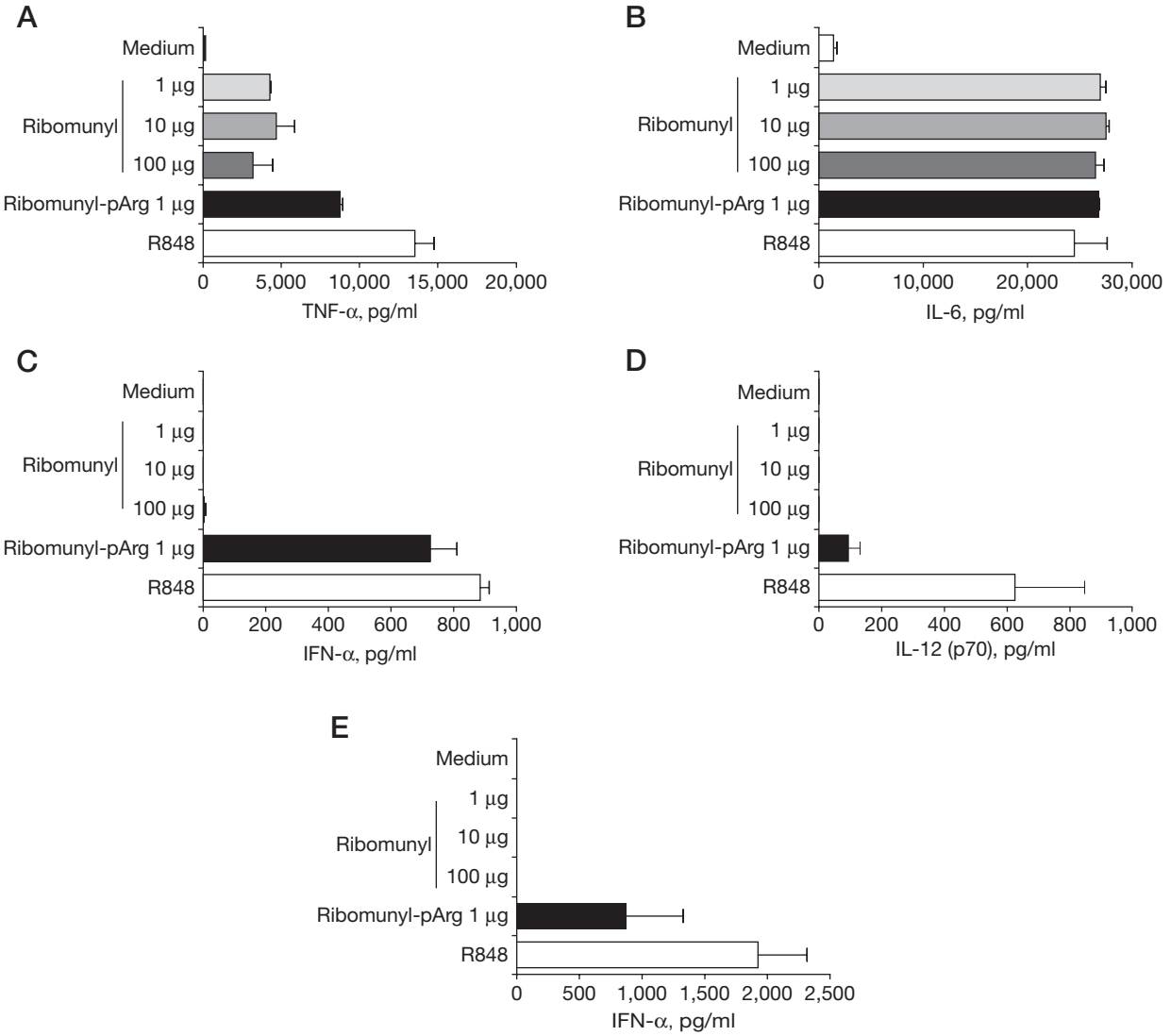
as stated. Results from all donors were normalized and compared by two-tailed unpaired Student's *t*-test (Prism 4; GraphPad Software, La Jolla, CA, USA; Additional file 1).

Results

Ribomunyl® activates IFN-α and IL-12 in PBMC and adenoid-derived immune cells after endosomal delivery

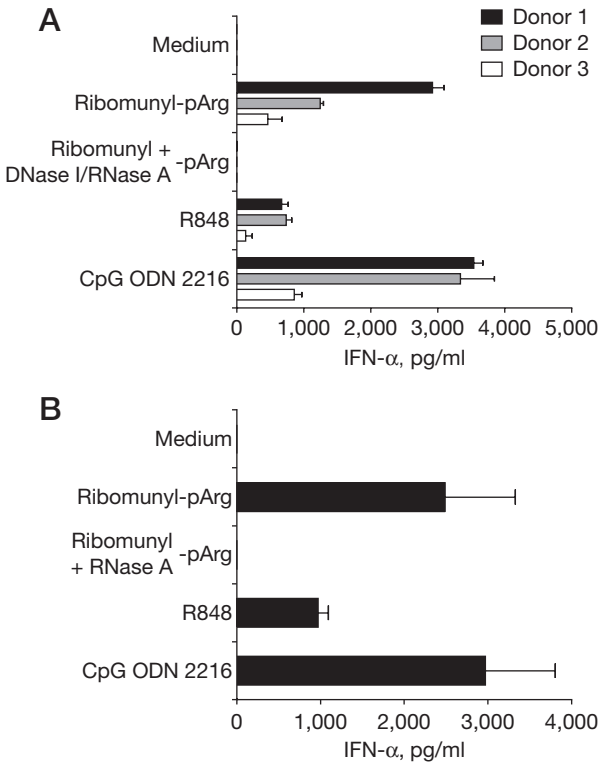
To evaluate the immunostimulatory capacity of Ribomunyl® *in vitro*, we analysed PBMC for cytokines induced by Ribomunyl®. Ribomunyl® is known to activate TLR2 [24–26], and as expected we observed substantial levels of the prototypic proinflammatory cytokines tumour necrosis factor-α and IL-6 induced by Ribomunyl® suspension (Figure 1A and 1B). By contrast, no antiviral type I IFN or IL-12 (p70) was detected even at high doses (100 μg/ml). Unlike TLR2, which is located on the cell surface, TLR7 and TLR8 detect pathogen RNA in the endosome of immune cells. We therefore delivered Ribomunyl® (1 μg/ml) in complex with pArg, and found that in this form

Figure 1. Ribomunyl® activates IFN-α secretion by PBMC and adenoid-derived immune cells exclusively after endosomal delivery



One representative result of three independent experiments is shown. Peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated with increasing concentrations of Ribomunyl® by itself or Ribomunyl® in complex with poly-L-arginine (pArg) or R848 (Toll-like receptor [TLR]7/8 ligand). After 24 h, supernatants were harvested and analysed by ELISA for **(A)** tumour necrosis factor (TNF)-α, **(B)** interleukin (IL)-6, **(C)** interferon (IFN)-α and **(D)** IL-12 (p70). **(E)** Immune cells from adenoids were stimulated as described above, and IFN-α was analysed by ELISA.

Figure 2. RNA is the immunostimulatory substance in endosomally delivered Ribomunyl®



Ribomunyl® was digested with **(A)** a mix of RNase plus DNase or **(B)** RNase alone and then formulated in complex with poly-L-arginine (pArg) to stimulate immune cells from adenoids. Ligands for Toll-like receptor (TLR)7/8 (R848) and TLR9 (CpG ODN 2216) were used as controls. After 24 h, supernatants were harvested and analysed for interferon (IFN)-α by ELISA. (A) Three different donors are shown. (B) One representative result of three independent experiments is shown.

Ribomunyl® induced substantial amounts of IFN-α (P<0.01 when compared with Ribomunyl® on its own, cumulative results from n=3 experiments), as well as IL-12 (p70; P<0.01, cumulative results, n=3) at levels comparable with the synthetic TLR7/8 stimulus R848 (Figure 1C and 1D). Similarly, Ribomunyl® was able to activate the immune cells from adenoids to secrete IFN-α in complex with pArg (Figure 1E; P<0.01, cumulative results, n=3). We conclude that when endosomal delivery is enhanced, Ribomunyl® can function as a potent antiviral immune stimulus even in virally colonized tissue.

RNA is the immunostimulatory substance in endosomally delivered Ribomunyl®

We then digested Ribomunyl® with RNase and/or DNase before transfecting it in complex with pArg into immune cells purified from adenoids (Figure 2A and 2B). Both the nuclease mix as well as RNase alone completely abolished the induction of IFN-α (P<0.01, cumulative results, n=3 each), indicating that the RNA contained in Ribomunyl® stimulated the IFN-α production.

Ribomunyl® acts as a combined TLR7 and TLR8 agonist with activity similar to synthetic RNA ligand 9.2s

Next, we aimed to identify the cell type within immune cells from adenoids and PBMC responsible for producing type I IFN in response to Ribomunyl®. Ribomunyl® in complex with pArg was able to induce IFN-α in total immune cells and in purified PDC from adenoids but not in adenoid immune cells depleted of PDC (Figure 3A; P<0.01, total immune cells versus total immune cells–PDC cumulative results, n=3). This indicates that PDC produce IFN-α upon Ribomunyl® stimulation. Overall, Ribomunyl® in complex with pArg induced IFN-α levels comparable with the strong PDC activator CpG DNA. We then purified RNA from Ribomunyl®. Agarose gel electrophoresis showed that RNA is present in Ribomunyl® but degraded during manufacturing (Additional file 2). For the reference RNA we chose the potent TLR7 and TLR8 agonist 9.2s RNA [30]. We stimulated PDC purified from PBMC, as well as PBMC depleted of PDC, with Ribomunyl® RNA by itself and in complex with pArg, as well as 9.2s RNA and CpG 2216 (Figure 3B and 3C). Only in complex with pArg did Ribomunyl® RNA induce IFN-α in PDC (P<0.01, cumulative results, n=3) and to a lesser extent IL-12 (p70) in PBMC without PDC (P<0.01, cumulative results, n=3), thus exhibiting an activation pattern of a combined TLR7 and TLR8 agonist.

Protamine, a polycation tested for RNA delivery in a clinical study, effectively delivers Ribomunyl® RNA to TLR7 and TLR8 of immune cells

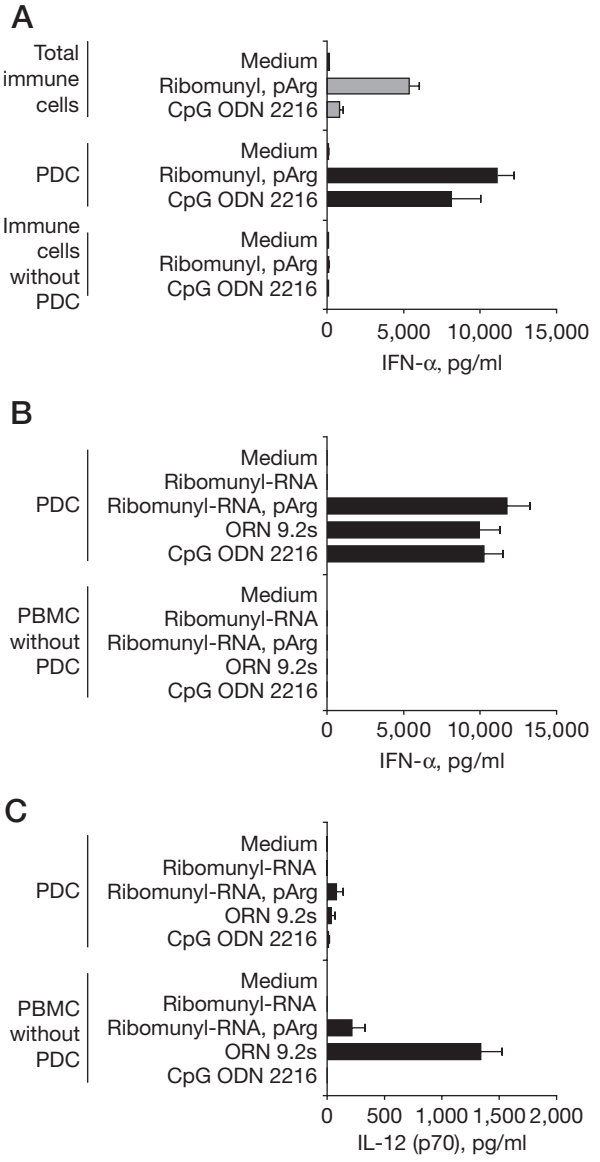
In contrast to pArg, the polycation protamine is approved for use in humans and has been shown to be safe and effective for the delivery of mRNA to TLR7 in a clinical study [32]. We therefore tested protamine for its ability to deliver Ribomunyl® RNA to TLR7 and TLR8 in our *in vitro* setting. In contrast to control RNA polyA, Ribomunyl® RNA in complex with protamine induced IFN-α in PBMC (Figure 4A; P<0.01, cumulative results, n=3) at amounts comparable with 9.2s RNA, and similarly in immune cells from adenoids (Figure 4B; P<0.01, cumulative results, n=3). The induction of IFN-α by Ribomunyl® in complex with protamine or pArg was dose dependent (Figure 4C). To confirm that Ribomunyl® RNA in complex with polycations indeed activated TLR7 and TLR8 we used HEK293 reporter cells. Selective activation of these cells was verified using the Imiquimod derivatives CI-087, CI-075 and R848 that activate TLR7, TLR8 or both, respectively (Figure 4D). Similarly, Ribomunyl® induced reporter

activity in TLR7-expressing and TLR8-expressing cells, but not in non-transgenic HEK293 cells (Figure 4E). Moreover, Ribomunyl® in complex with polycations was able to stimulate IFN-α release from bone marrow cells isolated from wild-type mice, but not from TLR7-deficient mice (Additional file 2), indicating that also in mice the IFN-α response to endosomally delivered Ribomunyl® was mediated by TLR7. Using a TLR3 reporter assay we found that neither Ribomunyl® alone nor Ribomunyl® in complex with polycations activated TLR3 (Additional file 2). However, congruent with previous publications [24–26], Ribomunyl® alone or in complex with protamine strongly activated TLR2 reporter cells. TLR2-dependent activation was reduced when RNA purified from Ribomunyl® was used, indicating partial removal of the TLR2 ligand *Klebsiella* OmpA (Figure 4F).

Discussion

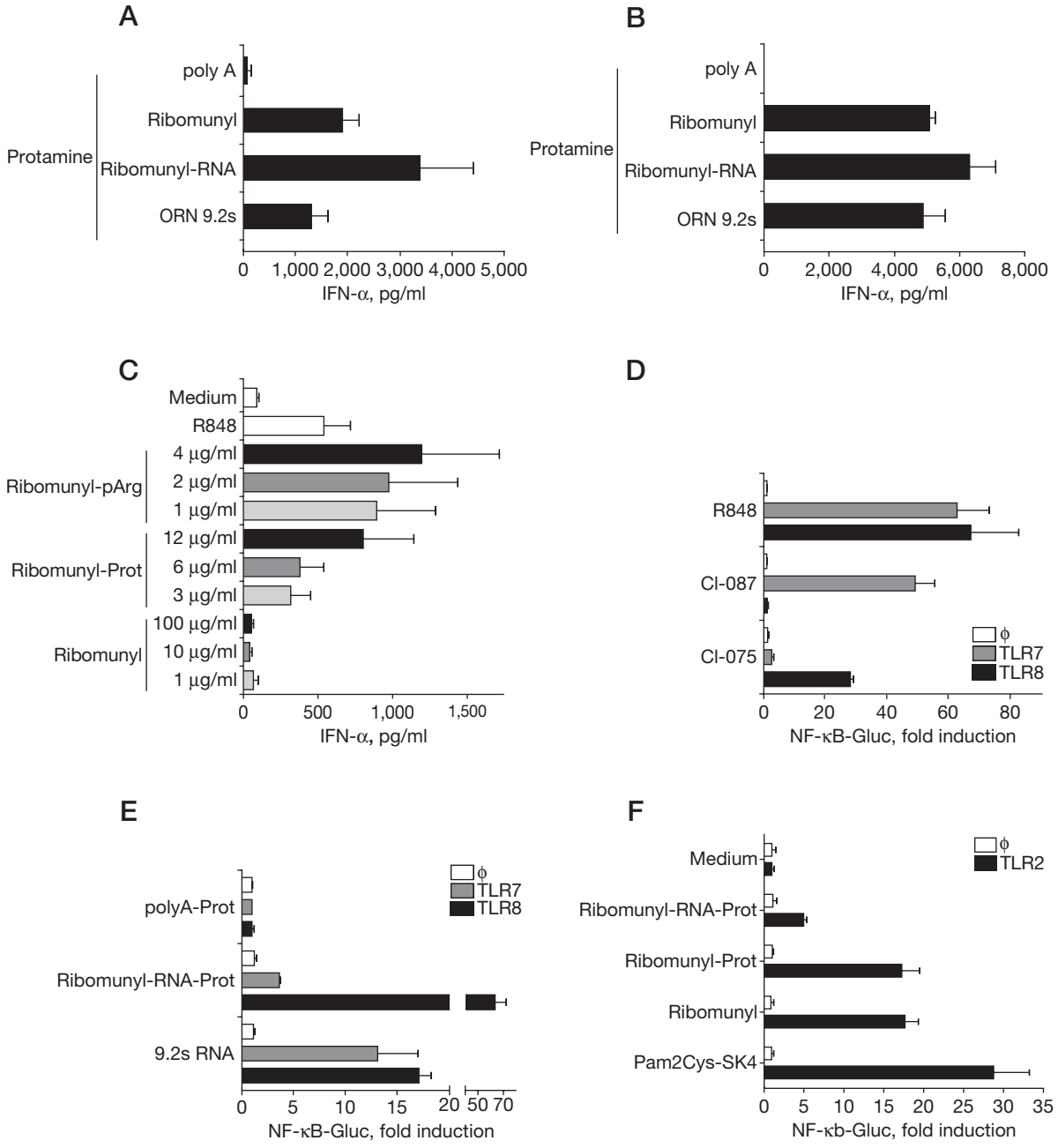
The innate immune receptors TLR7 and TLR8 recognize pathogen RNA and are able to induce an antiviral response. They are currently under investigation as targets for new antiviral drugs. TLR stimulatory drugs including CpG ODN or R848 have been shown to induce a robust activation of the innate immune system, and can safely be used in human therapy [33–36]. However, so far, none of these compounds have been clinically evaluated for the treatment of URTI. Because there is no virus-specific treatment available, new treatment options are needed. In contrast to synthetic RNA oligonucleotide ligands, which are still in pre-clinical development, Ribomunyl® has already been approved for human use in many countries. Some benefit in URTI has been reported, yet Ribomunyl® has never achieved broad clinical use. Nevertheless, these first-generation immunostimulatory drugs might be used to develop the concept of targeted delivery of TLR agonists to achieve immunostimulation in URTI. Therefore, in this work we analysed the immunostimulatory potential of Ribomunyl® on the innate immune system with a focus on its main component, bacterial ribosomal RNA. We demonstrate that if Ribomunyl® RNA is brought into the endosome by complex formation with polycationic proteins like protamine, it induces *in vitro* antiviral type I IFN and IL-12 (p70) via activation of TLR7 and TLR8 [37,38]. Ribomunyl® showed robust induction of IFN-α comparable with a synthetic TLR7/8 ligand 9.2s RNA. Ribomunyl® in complex with polycationic proteins was able to activate TLR7 and TLR8 not only within PBMC of healthy donors but in virally colonized upper respiratory tract lymphoid tissue, indicating that this combination might elicit antiviral activity even in the presence of a viral infection. By contrast, the proinflammatory cytokines IL-6 and tumour necrosis factor-α were detectable also without complex formation, most probably owing to TLR2 engagement by the *Klebsiella* proteoglycan KpOmpA contained in Ribomunyl® [24–26]. In summary, our data show that Ribomunyl® contains TLR2, TLR7 and TLR8 ligands, but does not significantly activate TLR3.

Figure 3. Purified Ribomunyl® RNA acts as a combined TLR7 and TLR8 agonist



One representative result of three independent experiments is shown. Total immune cells, purified plasmacytoid dendritic cells (PDC) and immune cells depleted of PDC, from **(A)** adenoids or **(B&C)** peripheral blood mononuclear cells (PBMC) were stimulated with Ribomunyl® or Ribomunyl® RNA by itself or in complex with poly-L-arginine (pArg). After 24 h, supernatants were harvested and (A&B) interferon (IFN)-α and (C) interleukin (IL)-12 (p70) concentrations were measured by ELISA. CpG ODN 2216 and 9.2s RNA were used as controls. ORN, oligoribonucleotide; TLR, Toll-like receptor.

Figure 4. Protamine, a polycation tested for RNA delivery in a clinical study, effectively delivers Ribomunyl® RNA to TLR7 and TLR8 of immune cells



Immune cells isolated from **(A)** adenoids or **(B)** peripheral blood mononuclear cells (PBMC) were stimulated with Ribomunyl®, purified Ribomunyl® RNA or 9.2s RNA in complex with protamine (Prot). After 24 h, supernatants were harvested and analysed for interferon (IFN)-α by ELISA. (A&B) One representative result of three independent experiments is shown. **(C)** PBMC were stimulated with increasing concentrations of Ribomunyl® alone or in complex with poly-L-arginine (pArg) or Prot as indicated. After 24 h, supernatant was harvested and analysed for IFN-α expression. Means of two independent donors are shown. **(D)** Toll-like receptor (TLR)7-expressing or TLR8-expressing 293 cells containing an NF-κB-reporter plasmid were stimulated with R848 (TLR7/8 ligand), Cl-087 (TLR7 ligand) and Cl-075 (TLR8 ligand). Luciferase induction after 16 h is shown as the fold increase over unstimulated cells. Means of two independent experiments are shown. **(E)** The 293 cells used in (D) were stimulated with polyA, 9.2s or Ribomunyl® RNA with Prot. Luciferase induction after 16 h is shown as the fold increase compared with polyA with Prot. Means of two independent experiments are shown. **(F)** The 293 cells expressing NF-κB-reporter and TLR2 or control plasmids were stimulated with TLR2 ligand Pam2Cys-SK4, Ribomunyl®, Ribomunyl® with Prot or Ribomunyl® RNA with Prot. Luciferase induction after 16 h is shown as the fold increase over unstimulated cells. Data shown are representative of three independent experiments. ORN, oligoribonucleotide; ϕ, untransfected.

These results raise the question of whether the current practice of oral administration of Ribomunyl® *in vivo* is optimal to limit URTI, because the potential antiviral activity via TLR7 and TLR8 engagement by the ribosomal RNA remains untapped.

Here, we evaluated protamine as an endosomal delivery agent *in vitro* because of properties that might prove advantageous for the transfer to an *in vivo* use. Protamine is already approved for therapeutic use in humans, and has been used safely and successfully for intracutaneous delivery of mRNA to TLR7 in dendritic cells of the skin in a Phase I/II clinical trial [32]. In addition, it is established for the delivery of messenger RNA/small interfering RNA in several animal models [39–41]. In the clinics, protamine is currently used intravenously to reverse the effect of heparin showing side effects including allergic reactions. Given that the upper respiratory tract is accessible for topical administration, this route appears preferable over a systemic administration to avoid potentially severe side effects. Also, local delivery to the adenoids requires much lower amounts of protamine than those currently used intravenously, again limiting possible side effects of protamine. Ribomunyl® in complex with microgram amounts of protamine was sufficient to induce IFN-α in cultured immune cells when delivered as an aerosol (Additional file 2), modelling application via spray to the immune cells in the upper respiratory tract. As an alternative to protamine, we are currently evaluating Eudragit® (Evonik Industries, Darmstadt, Germany) for RNA delivery that is used in drug formulation and is under investigation for mucosal drug delivery [42]. This approach should be applicable for a broad range of viruses because the antiviral mechanisms triggered by the innate immune system are not selective for any causative viral strain. Nevertheless, acute URTI characteristically has a short therapeutic window. Therefore, TLR7 stimulation holds promise particularly in recurrent disease and as prophylaxis.

This mechanistic study presented here is limited to an *in vitro* proof of principle that Ribomunyl® can act as a TLR7/8 agonist if delivered to the endosome preceding rigorous pre-clinical and clinical testing of the approach. Whether stimulating antiviral activity by aerosol delivery of Ribomunyl® in complex with protamine or defined synthetic ligands for TLR7 and TLR8 [18] to lymphatic tissues of the upper respiratory tract constitutes a safe and ultimately beneficial treatment option for URTI needs to be carefully evaluated in URTI animal models and additional clinical studies *in vivo*.

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Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: Supplementary Methods presenting further details on the procedures used can be found at http://www.intmedpress.com/uploads/documents/AVT-10-OA-1813_Herberhold_Add_file1.pdf

Additional file 2: Supplementary Figures presenting further results for Ribomunyl® can be found at http://www.intmedpress.com/uploads/documents/AVT-10-OA-1813_Herberhold_Add_file2.pdf

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