Plasmacytoid dendritic cell-derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists

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Abstract

There is a high demand for the development of adjuvants that induce cytotoxic T lymphocytes (CTL), which are crucial for the elimination of intracellular pathogens and tumor cells. Toll-like receptor (TLR) agonists are prime candidates to fulfill this role since they induce innate immune activation and promote adaptive immune responses. The successful application of the TLR7 agonist R837 for treatment of basal cell carcinoma shows the potential for exploiting this pathway in tumor immunotherapy. Imidazoquinolines like R837 and stimulatory single-stranded RNA (ssRNA) oligonucleotides both trigger TLR7-mediated immune activation, but little is known about their comparative ability to promote immunity induction. We investigated differences in innate immune activation and adjuvant activity between the imidazoquinoline R848 and the ssRNA TLR7 agonist polyUs21. In contrast to R848, polyUs21 induced detectable levels of intracellular interferon α (IFNα) in plasmacytoid dendritic cells (PDC). In immunisation studies, only polyUs21 led to robust priming of type 1 T helper (Th1) cells and CTL and it was more efficient in inducing anti-tumor immunity than R848. Notably, exogenous IFNα augmented the adjuvant activity of R848 while depletion of PDC abrogated the adjuvanticity of polyUs21. This study, therefore, identifies sufficient IFNα production by PDC as an important determinant of vaccine efficacy.
Introduction

Cellular immune responses characterized by the induction of cytotoxic effector cells are crucial for therapeutic interventions in the context of tumor immunotherapy and for the induction of protective immunity against a variety of intracellular pathogens such as the malaria parasite and human immunodeficiency virus. A particular focus of novel vaccination strategies is the identification of adjuvants with the ability to skew adaptive immune responses towards a Th1 phenotype and, thereby, allow for the induction of cellular, in addition to humoral immunity.\(^1\) Synthetic mimics of pathogen-associated molecular patterns (PAMPs) in general, and especially those mimicking virus presence appear particularly potent in promoting the induction of cellular immunity and might, therefore, constitute powerful adjuvants.\(^2\) Viral PAMPs can be detected by TLR and cytoplasmic pattern recognition receptors.\(^3,4\) The virus-sensing TLRs sample the contents of specialized endosomal compartments where they detect bacterial and viral genomes, as well as viral replication intermediates.\(^3,4\) Different classes of viral nucleic acids are detected by distinct TLRs with TLR3, TLR7/8 and TLR9 sensing double-stranded RNA, ssRNA and DNA, respectively.\(^3,4\)

Although various synthetic TLR agonists have been tried as adjuvants\(^5\), not many of them are approved for human use. In contrast, the TLR7/8 agonist R837 is approved for the topical treatment of genital warts, basal cell carcinoma and bladder cancer.\(^6-9\) Imidazoquinolines such as R837 and R848 were originally developed as small immune response modifiers with anti-viral activity and it only became evident later that they stimulate innate immune activation via TLR7 and/or TLR8.\(^10-13\) In mouse studies, imidazoquinolines were shown to act as adjuvants able to promote an adaptive immune response to co-administered antigens.\(^14,15\) However, R837 also leads to TLR7-independent augmentation of inflammation by acting as an adenosine receptor antagonist.\(^16\) Furthermore, repeated systemic administration results in immune dysfunction due to temporary depletion of peripheral leukocytes and altered lymphoid organ structure.\(^17,18\) Thus, systemic application of imidazoquinolines leads to adverse side effects\(^19\), and the development of other TLR7 agonists suitable for non-topical use as adjuvants is desirable. The identification of suitable TLR7 agonists requires a systematic comparison of these candidates with imidazoquinolines for the ability to promote adaptive immunity.
Various RNA oligonucleotides including siRNA constructs have the capacity to trigger TLR7. There is no clear consensus on the motif that mediates recognition via TLR7, with uridine- and GU-rich sequences having been proposed in addition to GU-independent motives. A limiting factor for TLR7-mediated immune activation by ssRNA is the access of the latter to the endosomal compartments, in which recognition takes place. Since free ssRNA is quickly degraded by extracellular RNases, ssRNA TLR7 agonists have to be used in the form of complexes with cationic compounds to be effective both in vitro and in vivo. However, this does not preclude their use in vivo and their potential as adjuvants for the induction of cytotoxic effector function has been suggested. Here, we explore the use of ssRNA TLR7 agonists as adjuvants for the induction of adaptive immunity. We demonstrate in a murine model system that a 21-mer of polyU previously reported to act as selective TLR7 stimulus is vastly superior to R848 at inducing CD4+ and CD8+ T cell responses to co-administered antigen. Notably, we show that this potency is attributable to its superior ability to stimulate IFNα production by PDC. This suggests that ssRNA TLR7 agonists could be developed as effective adjuvants for the induction of cellular immunity and that the ability to induce sufficient levels of IFNα is a crucial determinant for the selection of potent adjuvants.
Material and methods

Mice. C57BL/6 and 129 Sv mice were obtained from Harlan UK (Bicester, UK). TLR7 KO (C57BL/6 background) and IFNRI KO (129 Sv background) mice were bred at the biological service unit at King’s College London. All animal experiments were performed in accordance with UK governmental regulations (Animal Scientific Procedures Act 1986) and were approved by the UK Home Office.

Immunisation studies. For dendritic cell (DC) activation, mice were injected intravenously with 30µg of the indicated TLR agonists in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethansulfonic acid)-buffered saline (HBS) if not specified otherwise. PolyU (Sigma, Gillingham, Dorset, UK) and polyUs21 (provided by Innate Pharma, Dardilly, France) were administered in form of complexes with 100µl DOTAP (Roche, Burgess Hill, UK). For T and B cell activation, adjuvants were co-administered with 200µg of endograde ovalbumin (OVA) from Profos AG (Regensburg, Germany) or egg white preparation containing an equivalent of 600µg of OVA. Control mice received OVA in combination with DOTAP, but without TLR agonist. Exogenous IFNα (Hycult Biotechnology, Uden, Netherlands) and OVA were co-injected intravenously with or without R848 (Invivogen, Toulouse, France). As control, mice were treated with 30µg polyI:C (GE Healthcare, Chalfont St. Giles, UK) with or without OVA. In specified experiments, mice were immunised with OVA plus 15µg anti-CD40 antibody with or without R848 (BD Biosciences, Oxford, UK).

DC activation analysis. Splenocyte suspensions were stained with fluorochrome-labelled antibodies specific for mouse CD11c, CD4, CD8α, B220 (BD Biosciences) and PDCA1 (eBiosciences, Hatfield, UK). Intracellular cytokine staining of splenocytes was performed following a 3h-incubation in the presence of 5µM brefeldin A (Invitrogen, Paisley, UK). Cells were fixed in 4% paraformaldehyde and stained for the DC markers listed above. Subsequently, cells were permeabilized and stained for intracellular cytokines in PBS containing 1% heat-inactivated fetal calf serum, 5mM EDTA, 0.1% saponin (Sigma) and 0.02% sodium azide using PE-labelled IL-12p40- (clone C15.6; BD Bioscience), PE-labelled IL-6- (clone MP5-20F3; BD Bioscience) or
FITC-labelled IFNα-specific antibody (clone RMMA-1; PBL, Piscataway, NJ, USA). Data were acquired on a FACSCanto II (BD Biosciences).

**CTL priming.** On day 6 post vaccination, mice were injected intravenously with a 1:1 mixture of splenocytes that had been pulsed with 500nM SIINFEKL peptide or left unpulsed and labelled with 0.5µM and 5µM CFSE, respectively. The next day, splenocytes were isolated and analyzed by flow cytometry. Antigen-specific killing was calculated using the following formula: (1-% of CFSEpeptide/% of CFSENop peptide) x100.

**T helper cell and antibody responses.** On day 7 post vaccination, splenocytes were isolated and cultured *in vitro* in the presence or absence of 300µg/ml OVA for 72h. IFNγ expression was determined in cells incubated for an additional 3h with 5µM brefeldin A by intracellular cytokine staining with IFNγ-specific antibody (clone XMG1.2; BD Biosciences) as described above. Supernatants from these cultures were assayed for IFNγ, IL-5, IL-13 and IL-4 using FlowCytomix kits from Bender MedSystems (Vienna, Austria).

Relative levels of OVA-specific antibodies in the serum of mice vaccinated 7 days earlier were determined by ELISA using anti-mouse IgM (clone R6-60.2), IgG1 (clone A85-1) and IgG2c (clone R19-15) antibodies (all BD Biosciences).26

**Tumor model.** C57BL/6 mice were injected intravenously with 7.5x10⁵ B14.3 cells (B16 melanoma cells expressing OVA/GFP fusion protein) 30 days following prophylactic vaccination and the number of lung nodules was determined 18 days post tumor challenge. Up to 250 nodules were counted per lung.

**PDC depletion.** The hybridoma cell line for the monoclonal antibody 927 was kindly provided by Dr. M. Colonna, Washington University School of Medicine, St. Louis, MO, USA.27 The antibody was purified by protein G affinity chromatography. For depletion, mice were injected
intraperitoneally with 500µg of antibody 927 or control rat IgG2b antibody on two consecutive days. Mice were vaccinated 48h after start of the depletion. Depletion of PDC at the time of vaccination was confirmed by flow cytometry.

**Statistics.** Statistical analysis of *in vivo* CTL assays and the B16 metastasis model was performed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post test. Data on cytokine induction were analysed by using a two-tailed Student’s t-test.
Results

Stimulatory ssRNA and imidazoquinolines show differences in DC activation in vivo.

Adjuvants act in large measure by inducing the activation of DC, which is a prerequisite for induction of adaptive immunity. In order to compare the effects of distinct TLR7 agonists on DC subtypes *in vivo*, C57BL/6 mice were injected intravenously with polyU, polyUs21, R848 or the TLR7-independent control stimulus polyI:C. The imidazoquinoline R848 was chosen, since it induces stronger innate immune activation of DC than R837. Since ssRNA is prone to degradation by extracellular RNases, polyU and polyUs21 were administered in the form of complexes with the cationic lipid DOTAP. We determined the optimal ratio of ssRNA oligonucleotides and DOTAP for complex formation by dose titration (data not shown). A dose of 30µg polyU or polyUs21 complexed with 100µl of DOTAP led to maximal DC activation. For comparison, 30µg of R848 and polyI:C were administered without DOTAP, since at this dose robust innate immune activation was observed for both stimuli. *In vivo* activation of splenic DC was analysed 4h post TLR agonist administration by gating on CD11c int PDCA1+ B220+ PDC versus CD11c high conventional DC. Conventional DC were further subdivided into CD4+, CD8α+ and double-negative (DN) DC subsets. All four splenic DC subsets showed up-regulation of the maturation marker CD86 following injection of TLR agonists (Figure 1A). The extent of CD86 up-regulation on the different DC subsets was comparable between polyU, R848 and slightly lower for polyUs21 on conventional DC (Figure 1A). Furthermore, TLR7-mediated CD86 up-regulation was comparable to the levels observed in response to the TLR3 agonist polyI:C (Figure 1A). Similar patterns were obtained for up-regulation of CD40, another marker of DC maturation (Supplementary Figure 1A). Thus, all DC subsets underwent maturation in response to systemic treatment with the tested TLR agonists.

TLR7 and TLR3 are differentially expressed on mouse splenic DC with TLR7 being highly expressed by PDC and absent in CD8α+ DC and TLR3 showing the inverse expression pattern. Nevertheless, all DC subsets up-regulated CD86 in response to the TLR7 agonists indicating that activation in the case of TLR7-negative CD8α+ DC is indirect. While up-regulation of co-stimulatory molecules can be induced in a bystander fashion, cytokine secretion by DC is regarded as a sign of direct signalling by TLRs. As expected, all TLR7 agonists induced direct activation of
PDC, but the cytokine pattern that was induced varied (Figure 1B). Similar to earlier in vitro observations\(^{23}\), polyU and polyUs21 induced IFNα in PDC (6.94% ± 0.39 and 2.37% ± 0.49 IFNα\(^+\) PDC, respectively) whereas the imidazoquinoline R848 was ineffective in triggering detectable levels of IFNα (0.81% ± 0.49 IFNα\(^+\) PDC compared to 1.13% ± 0.40 IFNα\(^+\) PDC in mice treated with DOTAP only) in vivo (Figure 1B and Supplementary Figure 1B). In contrast, IL-12 p40 induction by PDC was similar or more pronounced in response to R848 (6.52% ± 2.81 versus 0.47% +/-0.19 IL-12 p40\(^+\) PDC in control mice) than upon treatment with the ssRNA TLR7 agonists (5.68% ± 2.02 versus 2.83% ± 0.65 IL-12 p40\(^+\) PDC for polyU and polyUs21, respectively). As expected PDC did not produce IFNα in response to the TLR3 agonist polyI:C (Figure 1B and Supplementary Figure 1B) and IFNα induction in response to polyU and polyUs21 administration was only detected in PDC and in none of the other DC subsets (data not shown).

The percentage of IL-12 p40-producing CD4\(^+\) and DN DC was increased in response to the TLR7 agonists with R848 stimulating more DC to produce this cytokine than polyU or polyUs21 (Figure 1C). PolyUs21 was less potent in activating TLR7-expressing DC subsets than polyU in inducing IL-12 p40. The TLR3 agonist polyI:C failed to induce IL-12 p40 in CD4\(^+\) and DN DC, but led to an increase in IL-12 p40-expressing CD8α\(^+\) DC (Figure 1C). Unexpectedly, there was a clear increase in the number of CD8α\(^+\) DC, which produced IL-12 p40 in response to the TLR7 agonists polyU, polyUs21 and R848 (Figure 1C). It is currently unclear whether the induction of IL-12 p40 by CD8α\(^+\) DC in response to TLR7 agonists is TLR7-dependent and is mediated via direct or indirect activation of this TLR7-negative DC subset. Direct activation of CD8α\(^+\) DC by R848 has not been observed in vitro suggesting that other cell types may be involved in the induction of IL-12 p40 by CD8α\(^+\) DC in vivo.\(^{28}\)

Thus, differences in TLR7-induced DC activation between ssRNA agonists versus imidazoquinolines that had been observed in vitro\(^{23}\) are also discerned following TLR7-mediated stimulation of splenic DC in vivo. For the subsequent detailed analysis of the adjuvanticity of TLR7 agonists, R848 was compared to polyUs21. Despite its lower activity as an innate stimulus for DC activation, polyUs21 was favoured over polyU, because it is of defined length and shows lower susceptibility to degradation by RNases. Additionally, polyUs21 is entirely endotoxin-free.
Differential adjuvanticity of distinct TLR7 agonists.

To determine how differences in innate immune activation by different TLR7 agonists influence the induction of adaptive immune responses, we co-injected TLR7 agonists with OVA to study their adjuvant properties. Splenocytes were isolated 7 days following immunisation and were re-stimulated with antigen in vitro. Culture supernatants of re-stimulated splenocytes from polyUs21- and polyI:C-treated mice revealed high levels of IFNγ, in contrast to splenocytes from R848-treated mice (Figure 2A). IFNγ production was dependent on re-stimulation and was not observed in the absence of antigen (Figure 2A). We were unable to detect the Th2 cytokines IL-4, IL-5 and IL-13 in re-stimulated splenocyte cultures (data not shown).

To determine whether IFNγ was produced by CD4+ T cells, activation of re-stimulated splenocytes was assessed by intracellular cytokine staining gating on CD4+ CD3+ cells. Following immunisation with antigen plus polyUs21 or polyI:C, the frequency of IFNγ-producing CD4+ T cells was elevated upon re-stimulation from 0.87% in the DOTAP control group to 3.38% and 2.28%, respectively (Figure 2B). In contrast, re-stimulated splenocytes from R848-treated mice showed only a slight increase in IFNγ-producing CD4 T cells to 1.37% (Figure 2B). In the absence of re-stimulation, the frequency of IFNγ-positive CD4 T cells was low and ranged from 0.35% to 0.53% for the different treatment groups (Figure 2B). In conclusion, treatment with polyUs21 complexed to DOTAP induces a Th1 response similar to the control adjuvant polyI:C, whereas R848 is relatively inefficient in priming this type of T helper cell response.

Effective priming of CTL is crucial for the induction of cellular immunity. We quantified the induction of CTL in response to the different adjuvants by performing an in vivo CTL assay. Immunisation with OVA plus polyUs21-DOTAP complexes or polyI:C led to potent CTL responses whereas OVA plus R848 failed to induce levels of CTL killing above the background levels observed for mice injected with DOTAP and OVA alone (Figure 2C). In order to rule out the possibility that the observed lack of in vivo CTL killing activity with R848 was the result of administering a suboptimal dose, a dose titration was performed. None of the tested doses of R848 (0.01-100µg/mouse) led to reproducible induction of CTL activity whereas titration of polyUs21
(0.1-100µg/mouse) showed a clear dose response for CTL induction (Figure 2D). Co-administration of R848 with DOTAP as performed for polyUs21 also failed to improve the induction of CTL responses (data not shown). In contrast, co-administration of anti-CD40 antibody, which mimics T cell help, increased levels of CTL induction by R848 (Supplementary Figure 2).

We also analyzed the humoral response potentiated by these adjuvants. We determined the levels of OVA-specific antibodies by ELISA and analysed IgM, IgG1 and IgG2c isotypes separately. Interestingly, immunisation with OVA plus polyUs21-DOTAP complexes led to high antibody titres whereas OVA plus R848 induced relatively low titres for all three isotypes (Figure 2E). A clear preference for class switching to IgG1 versus IgG2c antibodies could not be observed for either of the TLR7 agonists. In contrast to our findings, the ssRNA TLR7 agonist RNA40 has been shown to preferentially induce IgG1 antibodies upon subcutaneous administration. The discrepancy in the preference of B cell class switching induced by these two ssRNA TLR7 agonists could be either caused by differences in their stimulatory activity or the route of administration. The control adjuvant polyI:C induced relatively low IgG1 in comparison to IgG2c antibody titers (Figure 2E), thereby displaying a preference for Th1-dependent class switching.

Our results suggest that the observed differences in DC-derived cytokine induction upon systemic administration of polyUs21 versus R848 result in differences in priming of both CD4+ and CD8+ T cell responses. In addition, R848 is a poor inducer of B cell class switching in direct comparison to polyUs21, leading to a weaker humoral response. In summary, polyUs21 represents a more reliable and potent adjuvant upon systemic administration.

Induction of an anti-tumor immune response upon immunisation with TLR7 agonists.

To evaluate the adjuvant activity of the ssRNA TLR7 agonist polyUs21 versus the imidazoquinoline R848 for induction of anti-tumor immunity, we assessed them in a prophylactic vaccination regimen in a mouse model of lung metastases. Mice were given OVA plus different TLR agonists and were challenged intravenously with OVA-expressing B16 melanoma cells 30 days post vaccination after the primary response had waned. The development of lung nodules was determined 18 days after tumor challenge. Control mice that had been treated with OVA and
DOTAP in the absence of adjuvant were partially protected from tumor challenge, in contrast to mice injected with buffer alone (Figure 3). Mice vaccinated with OVA in combination with polyUs21-DOTAP complexes were completely protected from metastases as were mice that received OVA in combination with polyI:C (Figure 3). Mice vaccinated with a combination of OVA and R848 were only partially protected from tumor growth. Thus, while R848 can boost the induction of anti-tumor immunity, ssRNA agonists for TLR7 are more effective as adjuvants for generation of a protective anti-tumor immune response. The fact that any tumor protection is seen in R848-treated mice is surprising in view of the poor CTL induction. However, elimination of tumor cells in OVA plus R848-treated mice could be mediated by classes of cytotoxic effector cells other than CTL such as NK cells or cytotoxic DC.29,30 Future studies will have to clarify the mechanisms by which these different TLR7 agonists lead to the killing of tumor cells.

The adjuvant activity of polyUs21 is TLR7-dependent.

In order to determine whether the adjuvant activity of polyUs21 is exclusively mediated via TLR7, we evaluated the in vivo CTL killing responses in TLR7-deficient (TLR7 KO) mice. The CTL response in polyI:C-treated TLR7 KO mice was undiminished (Figure 4A). In contrast, the level of antigen-specific target cell killing in TLR7 KO mice immunised with polyUs21-DOTAP complexes was reduced to background levels, indicating that the adjuvant activity of polyUs21 is TLR7-dependent (Figure 4A). Similarly, the generation of antigen specific antibodies in response to polyUs21 as adjuvant was strongly inhibited in TLR7 KO mice whereas antibody production in polyI:C-treated TLR7 KO mice was only marginally reduced (Figure 4B). Surprisingly, low IgG1 antibody titres above the levels seen for OVA plus DOTAP-treated control mice were still detected in TLR7 KO indicating the presence of residual TLR7-independent Th2-skewing adjuvant activity.

The difference in adjuvant activity between polyUs21 and R848 is caused by differences in type I IFN (IFN-I) induction.

The results presented thus far provide evidence that ssRNA agonists are effective adjuvants whereas imidazoquinolines are less potent upon systemic administration. Based on the DC-derived
cytokine induction patterns observed \textit{in vitro}\textsuperscript{23} and \textit{in vivo} (Figure 1), we hypothesised that differences in the ability to induce IFN-I could be one of the factors that determine the adjuvanticity of TLR7 agonists. In order to test this hypothesis the induction of CTL responses was assessed in IFN-I receptor-deficient (IFNRI KO) mice. Interestingly, antigen-specific killing of target cells observed in 129 Sv WT mice was completely abolished in IFNRI KO mice irrespective of whether polyUs21 or polyI:C was used as adjuvant (Figure 5A). This indicates that signalling through the IFN-I receptor is indeed crucial for CTL priming \textit{in vivo} in response to immunisation with antigen plus TLR agonists. IFN-I acts on a variety of cell types and promotes cross-priming of CTL via direct and indirect mechanisms.\textsuperscript{31,32} Furthermore, the failure to mount anti-viral CTL responses in IFNRI KO mice has been attributed to defects in the survival of antigen-specific CD8 T cells upon priming.\textsuperscript{33}

Since R848 was found to be ineffective in inducing detectable levels of intracellular IFN\(\alpha\) in PDC we tested whether co-injection of IFN\(\alpha\) with antigen and R848 enables CTL cross-priming. Administration of antigen alone or in combination with either IFN\(\alpha\) or R848 induced minimal to low antigen-specific killing responses (Figure 5B). In contrast, co-administration of IFN\(\alpha\) and R848 together with antigen enhanced OVA-specific CTL activity (Figure 5B). The amplification of CTL induction by IFN\(\alpha\) was dose-dependent and administration of \(10^5\) U of IFN\(\alpha\) per mouse significantly increased CTL activity (Figure 5B). These results indicate that exogenous addition of IFN\(\alpha\) during priming can compensate for the inability of R848 to induce sufficient levels of this cytokine.

IFN\(\alpha\) induced in response to TLR7-mediated activation is exclusively PDC-derived (Figure 1). We, therefore, examined the effect of PDC-depletion on the induction of CTL responses in polyUs21-treated mice. Depletion of PDC was carried out using a PDC-depleting antibody (clone 927) and confirmed by flow cytometry on day 1 and 3 post immunisation (data not shown). PDC-depleted mice showed a significant reduction in the level of antigen-specific killing compared to mice treated with isotype control antibody (Figure 5C). The ability of polyI:C to boost CTL priming was found to be unaltered by PDC depletion, in accordance with the ability of polyI:C to induce IFN-I in cell types other than PDC via TLR3-dependent and -independent mechanisms.\textsuperscript{3} In contrast, for TLR7-targeting vaccines, PDC activation is a key component of the immune response.
Discussion

Adjuvants are crucial for the induction of antigen-specific immune responses and determine the qualitative and quantitative phenotype of the adaptive immune response to a co-administered antigen. Therefore, the development of molecularly defined adjuvants such as TLR agonists for vaccine design will benefit from understanding the mechanisms of their adjuvanticity. In the present study, we qualitatively and mechanistically investigated the adjuvant activity of the ssRNA TLR7 agonist polyUs21 in comparison to the TLR7-activating imidazoquinoline R848 and the TLR3 agonist polyI:C. Our data demonstrate the potent adjuvanticity of polyUs21. When used in combination with antigen, polyUs21 promotes the induction of a Th1 response, CTL cross-priming, B cell class switching and is effective in the initiation of anti-tumor immunity. Our study indicates that the adjuvant activity of polyUs21 is dependent on PDC-derived IFN-I suggesting that the development of TLR7 agonists as adjuvants needs to focus on the induction of a systemic IFN-I response in addition to the phenotypic and functional activation of professional antigen-presenting cells such as DC. Whether PDC are required solely for production of systemic IFN-I or also play a crucial role in antigen-presentation and cross-priming of CTL in response to immunization with antigen and ssRNA TLR7 agonists is currently unclear and awaits further dissection of the underlying mechanisms of TLR7-mediated immunity induction.

This is the first report of a direct and systematic comparison of imidazoquinolines versus ssRNA TLR7 agonists with regard to quantitative and qualitative differences in innate immune activation and adjuvanticity upon systemic administration in vivo. In previous studies, imidazoquinolines such as R848 have been shown to induce IFN-I in vitro and in vivo\textsuperscript{11,12,34-37}, which seems to contradict our finding that R848 is inefficient in inducing IFN\(\alpha\). However, there are differences in the frequency of DC between different mouse strains, which affect the serum levels of IFN-I that are observed upon TLR7 stimulation and C57BL/6 mice as used in this study have a low frequency of these IFN-producing cells.\textsuperscript{38} Another contributing factor that may lead to discrepancies in IFN-I induction between different studies is the sensitivity of different IFN\(\alpha\)-detecting methods, which varies widely. While the bioassay used in many of these previous studies is the most sensitive method for IFN-I detection\textsuperscript{11,34,37}, detection of IFN\(\alpha\) by ELISA is less robust, in particular for serum samples\textsuperscript{39}, and detection by intracellular staining requires high levels of
IFNα to be present in the cells. For the present study, we also have tried to quantify IFNα by ELISA, yet serum levels of IFNα were very low to undetectable in all mice, including polyI:C- and polyUs21-treated mice, underlining the insufficient sensitivity of this method. Thus, R848 may induce low levels of this cytokine, which fall below the detection threshold of the intracellular cytokine staining in our experiments. In accordance with this interpretation of the data, we reported in a previous in vitro study that R848 induces 30 times less IFNα than polyUs21.23

Despite the fact that IFNα was not detected in response to systemic administration of R848 in our study, we observed clear innate immune activation of various DC subsets including PDC on the level of IL-12 p40 induction and up-regulation of co-stimulatory molecules. The frequency of IL-12 p40-producing DC was even higher for R848- than for polyUs21-treated mice. Nevertheless, innate immune activation induced by R848 provided insufficient adjuvant activity as demonstrated by its failure in inducing CTL responses and its weak induction of T helper cells and antibody production. R848 also induced partial protection from tumor challenge underlining that it is not entirely devoid of adjuvant activity, but that it compares unfavorably with the nucleic acid TLR7 agonist polyUs21 and the dsRNA mimic polyI:C. While imidazoquinolines such as R848 have been shown to be poor adjuvants40-42, this study shows for the first time that the nucleic acid TLR7 agonist polyUs21 has potent adjuvant activity upon systemic administration, which is comparable to the adjuvanticity of the viral dsRNA mimic polyI:C.

A recent study comparing different TLR agonists has identified polyI:C to be the most effective adjuvant for induction of Th1 responses.15 The study demonstrates that polyI:C induces higher levels of IFN-I than topically applied R848 and that the adjuvant activity of polyI:C is crucially dependent on IFN-I induction.15 Our data support the conclusion that the induction of IFN-I is crucial for adjuvant-induced induction of Th1 responses and CTL cross-priming. While IFN-I in response to polyI:C is produced by cells of hematopoietic and non-hematopoietic origin15, PDC are the exclusive source of TLR7 agonist-induced IFN-I. Our study demonstrates that the ssRNA TLR7 agonist polyUs21, in contrast to R848, is a potent inducer of PDC-derived IFN-I upon intravenous administration and that its adjuvant activity is similar to that of polyI:C. Thus, ssRNA TLR7 agonists hold great potential to be developed as adjuvants in parallel to polyI:C. Since there are fundamental differences in the cell types that directly respond to polyUs21 versus
polyI:C and in the signaling pathways that are triggered, the application of these adjuvants could be complementary allowing for synergy of the different innate activation pathways. For the development of TLR7 agonists such as polyUs21 as adjuvants, several technical aspects have to be considered and potentially improved. The stability of ssRNA oligonucleotides is much lower than that of DNA oligonucleotides and even though the introduction of phosphorothioate bonds decreases their susceptible to degradation, polycationic compounds such as DOTAP are still required to ensure efficient delivery and TLR7 activation. However, additional modifications of the ssRNA oligonucleotides that increase their stability allow for their use in the absence of nucleic acid-condensing reagents in vivo. Alternatively, the development and optimization of new compounds for binding and condensation of ssRNA TLR7 agonists could improve their adjuvanticity for in vivo application. When developing new reagents for complexing ssRNA TLR7 agonists it also has to be taken into account that complex formation between immunostimulatory nucleic acids and cationic compounds has been shown to influence endosomal maturation and, thereby, affects TLR-mediated signaling and cytokine induction. The translation of findings on TLR7-mediated responses from the mouse model to the human system is complicated by the fact that murine TLR8 is non-functional or does not contribute to immune activation by ssRNA agonists and imidazoquinolines under the usual experimental conditions. In contrast to the mouse model where ssRNA-mediated immune activation is restricted to TLR7, in humans TLR8 has an overlapping yet distinct sensitivity. Both R848 and ssRNA oligonucleotides including polyUs21 are agonists for TLR7 and TLR8 in the human system. Taking this into account, it is very likely that ssRNA TLR agonists such as polyUs21 also act as potent adjuvants in the human system.

In summary, our data provide evidence that differences in the innate cytokine induction pattern between different classes of TLR7 agonists affect their ability to act as adjuvants. Our results point out the crucial role of PDC-derived IFN-I in skewing and boosting adaptive immunity in response to TLR7 agonists and demonstrate that polyUs21 is a potent TLR7-dependent adjuvant with the capacity to prime effective CTL responses. We conclude that polyUs21 and similar
immunostimulatory ssRNA oligonucleotides represent potent candidates for the development of adjuvants for tumor immunotherapy and for vaccination against pathogens.
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Authorship

Contribution: D.R. and S.S.D. designed and performed experiments, analyzed data and generated the figures; C.P. and Y.M. provided supporting data and advised on study design and manuscript layout; S.U. and S.A. contributed genetically modified mice; S.S.D. wrote and edited the manuscript.

Conflict-of-interest disclosure: The authors declare a conflict of interest. C.P. and Y.M. are employees of Innate Pharma, which has a financial interest in TLR7 agonists. S.S.D. and C.P. are inventors on a patent application relating to TLR7 agonists, which has been licensed to Innate Pharma from Cancer Research Technology.

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References


Figure Legends

Figure 1: ssRNA induces efficient DC activation in vivo. C57BL/6 mice were injected intravenously with the indicated TLR agonists. PolyU and polyUs21 were administered as complexes with DOTAP. Splenocytes were isolated and DC populations were analysed by flow cytometry. (A) The up-regulation of the maturation marker CD86 on gated DC subsets was determined 4h post adjuvant administration. (B) The percentage of IFNα and IL-12p40 expressing PDC was assessed by flow cytometry 4h post treatment. (C) The IL-12 p40 expression by PDC, CD4+, DN and CD8α+ DC was analysed as in B. The data are representative of three independent experiments (A, B) or are compiled to one figure with the SEM depicted (C).

Figure 2: polyUs21 induces potent T and B cell responses in vivo. C57BL/6 mice were immunised intravenously and 7 days later T and B cell responses were analysed. (A) Splenocytes were isolated and cultured in vitro for 72h in the presence or absence of OVA. IFNγ levels in the culture supernatants were determined by bead-based ELISA assay. Data contain results from at least two independent experiments. (B) IFNγ production by splenocytes cultured for 72h in the presence or absence of OVA was assessed by flow cytometry gating on CD4+ CD3+ T cells. One representative of three independent experiments is shown. (C) In vivo CTL killing assays were performed at day 7 post immunisation. Pooled data of two independent experiments are shown. (D) The indicated doses of R848 or polyUs21 in form of complexes with DOTAP were administered intravenously in combination with egg white preparation (n=3). In vivo CTL assay was performed 7 days post immunisation. (E) OVA-specific antibody levels of IgM, IgG1 and IgG2c isotypes in serial dilutions of serum were determined by ELISA in mice treated with adjuvant (solid lines) or in OVA +DOTAP treated control mice (dotted lines). Serum dilution curves represent the mean +SEM of at least three independent experiments.

Figure 3: Prophylactic vaccination with polyUs21 prevents tumor growth. C57BL/6 mice were challenged intravenously with B14.3 melanoma cells 30 days following vaccination with OVA in combination with the indicated TLR agonists. Control mice were injected with HBS or with a mixture of OVA and DOTAP. The tumor burden was determined 18 days post tumor challenge. Pooled data from at least three independent experiments are shown.
Figure 4: Adaptive immune responses induced by immunisation with polyUs21 are TLR7-dependent. (A) C57BL/6 and TLR7 KO mice were immunised with a combination of OVA and the indicated TLR agonists. PolyUs21 was administered in form of complexes with DOTAP and control mice received a combination of OVA and DOTAP. In vivo CTL assays were performed 7 days post immunisation. Data are a pooled from four independent experiments. (B) OVA-specific serum titres of IgG1 and IgG2c antibodies were determined by ELISA in WT and TLR7 KO mice immunised with (solid lines) or without (dotted lines) TLR agonists. Serum dilution curves represent the compiled data of three independent experiments with the SEM indicated.

Figure 5: CTL responses to polyUs21 are dependent on signalling through IFNRI. (A) 129 Sv mice and IFNRI KO were vaccinated with egg white preparation as a source of OVA in combination with the indicated TLR agonists. In vivo CTL killing assays were performed at day 7 post vaccination. Data are pooled from two independent experiments. (B) C57BL/6 mice were treated with egg white preparation in combination with IFNα (10⁴ or 10⁵ U/mouse) either in the absence or presence of 100µg R848. On day 7 post vaccination, the in vivo CTL killing assay was performed. Data show the mean +SEM from three independent experiments. (C) C57BL/6 mice were treated with PDC-depleting (clone 927) or isotype control antibody. Upon depletion of PDC, mice were vaccinated with OVA in combination with the indicated TLR agonists. The in vivo CTL killing assay was performed at day 7 post vaccination. The graph contains pooled data from three independent experiments with the SEM indicated.
Figure 1
Figure 2
Figure 4

(A) Specific killing (%) of WT and TLR7 KO cells treated with different lipids.

(B) Absorbance at 405 nm for WT and TLR7 KO cells with different serum dilutions and antibody treatments.
Figure 5
Plasmacytoid dendritic cell-derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists

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