Small molecule Toll-like receptor 7 agonists localize to the MHC class II loading compartment of human plasmacytoid dendritic cells

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Abstract

Toll-like receptors (TLRs) 7 and 8 are intracellular sensors activated by single stranded RNA species generated during viral infections. Various synthetic small molecules can also activate TLR7 and/or 8 through an unknown mechanism. Notably, direct interaction between small molecules and TLR7 or 8 has never been demonstrated. To shed light on how small molecule agonists target TLRs, we labeled two imidazoquinolines, resiquimod and imiquimod, and one adenine-based compound, SM360320, with two different fluorophores (TAMRA and AlexaFluor488) and monitored their intracellular localization in human plasmacytoid dendritic cells (pDCs). All fluorescent compounds induced the production of IFN-α, TNF-α and IL-6 and the up-regulation of CD80 and CD86 by pDCs showing they retained TLR7 stimulating activity. Confocal imaging demonstrated that all compounds concentrated in the major histocompatibility complex class II loading compartment (MIIC), identified as lysosome associated membrane protein (LAMP)-1⁺, -3 (CD63)⁺ and human leukocyte antigen (HLA)-DR⁺ endosomes, of pDCs, like CpG-B. Treatment of pDCs with bafilomycin A, an antagonist of the vacuolar-type proton ATPase controlling endosomal acidification, prevented the accumulation of small molecule TLR7 agonists, but not of CpG-B, in the MIIC. These results indicate that a pH-driven concentration of small molecule TLR7 agonists in the MIIC is required for pDCs activation.
Introduction

Since the discovery of type I interferon (IFN-I) in 1957 and its potential for treatment of viral infections and cancer, several small molecule inducers of this factor have been identified. Many of these compounds are now known to be Toll-like receptor (TLR) 7 and/or 8 agonists. Among them are imidazoquinolines, like resiquimod (R848) that activates both TLR7 and 8 in humans, and imiquimod (R837) that activates TLR7 only. Imidazoquinolines have been largely tested in humans and R837, formulated as a cream (Aldara™), is licensed for topical treatment of genital warts, basal cell carcinoma and actinic keratosis. Similar to R837, purine-like molecules such as 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (SM360320 or 1V136), are TLR7 agonists. TLR7 and 8 are phylogenetically related, located on the X chromosome in most mammals and likely derive from a gene duplication event. TLR7 and 8 are both activated by various single stranded (ss)RNAs from viruses, synthetic guanosine- or uridine-rich ssRNAs, and synthetic small molecules. TLR9, together with TLR7 and 8, form a subfamily of TLRs based on genomic structure and sequence homology. The natural agonist of TLR9 is unmethylated, CpG-containing DNA of bacterial or viral origin that can be mimicked by synthetic ssCpG oligonucleotides that can be classified as A-type (CpG-A), B-type (CpG-B) or C-type (CpG-C) based on their different sequence motifs and biological activities. Several reports have suggested that TLR7, 8 and 9 can interact directly with nucleic acids, however, structural evidence confirming this hypothesis is not available yet. No convincing evidence of direct binding of imidazoquinolines or purine-like molecules to TLR7 or 8 exists either.

TLR7 and 9 are the only TLRs expressed by plasmacytoid dendritic cells (pDCs), which are a rare subset of circulating DCs that are considered as a front line defense against viral infections. pDCs produce most of the systemic IFN-I (i.e. IFN-α and -β) following viral infection and rapidly activate T cells utilizing pre-synthesized MHC class I and II molecules stored in their early and late endosomal
Although nucleic acid sensing provides protection from intracellular infection, nucleic acids are not exclusively from pathogens and therefore, a balance between self versus foreign responsiveness is necessary. Indeed, recognition of self nucleic acids by TLR7 and 9 and the resultant IFN-I production have been linked to autoimmune disorders such as lupus \(^{18}\). To avoid autoimmunity driven by recognition of self nucleic acids, TLR3, which binds double stranded RNA \(^{19}, 7, 8 \text{ and } 9\) are sequestered in intracellular compartments. In particular, TLR7 and 9 localize to the endoplasmic reticulum and the endolysosomal compartment, which is a still incompletely understood vesiculotubular network of sorting and degradative organelles that accept, redirect and process molecules from the exterior or interior of the cell \(^{20}\). TLR7 and 9 translocation from the endoplasmic reticulum to the endolysosomal compartment depends on the chaperone protein UNC93B and is required for TLR7 and 9 signaling \(^{21,22}\). Recent evidence shows that TLR9 becomes competent for signaling upon proteolytic cleavage of its ectodomain in the endolysosomal compartment \(^{23-26}\). The need for proteases in intracellular TLR activation may be a common phenomenon since cleaved TLR7 has been found in RAW264.7 cells \(^{23}\) and Sepulveda et al. claimed that human TLR7 is cleaved in DCs upon R837 stimulation, possibly by asparagine endopeptidase, like TLR9 \(^{25}\). Furthermore, several reports have shown that CpG concentrates in the endolysosomal compartment \(^{13,27,28}\), supporting the idea that the productive encounter between TLR9 and its ligand occurs there. In particular, localization of CpG-A to transferrin receptor (TfR)\(^+\) endosomes was associated with IFN-\(\alpha\) production, while localization of CpG-B in lysosome-associated membrane protein (LAMP)-1\(^+\) endosomes was associated with co-stimulatory molecule (e.g. CD80 and CD86) up-regulation in human pDCs \(^{28}\).

Unlike CpG, the intracellular localization of small molecule TLR7/8 agonists has not been characterized. Thus, we labeled R848, R837 and SM360320, with fluorophores to track them in human pDCs. All three fluorescently labeled compounds were TLR7 agonists and induced IFN-\(\alpha\), TNF-\(\alpha\) and
IL-6 production as well as and CD80 and CD86 up-regulation by human pDCs, like their unlabeled parent compounds and CpG-B, as described 28-30. Fluorescently labeled compounds co-localized with CpG-B in LAMP1+, lysosome-associated membrane protein-3 (CD63)+ and human leukocyte antigen (HLA)-DR+ endosomes, which belong to the MHC class II loading compartment (MIIC) of pDCs, independently of their chemotype and of the fluorophore used to label them. Bafilomycin A, an antagonist of the vacuolar type proton ATPase responsible for endosomal acidification 31, strongly inhibited the localization of the TLR7 agonist small molecules while leaving unaltered the localization of CpG-B. We conclude that the acidic pH present in the MIIC is the driving force for small molecule TLR7 agonists localization and a key requirement for their immunostimulatory activity.
Materials and Methods

Fluorescently labeled TLR7 agonist small molecules and CpGs.

Synthesis of R848, R837 and SM360320, fluorescently labeled or not, is described in Supplemental data. CpG-B (2006): 5’- tcg tcg ttt tgt cgt ttt gtc gtt -3’ and CpG-A (D19): 5’-ggTGCATCGATGCAGggggg-3’, labeled or not at the 3’-end with 5(6) carboxytetramethylrhodamine (TAMRA), were from Primm. CpG-B-FITC was from Invivogen. Bases shown in capital letters are phosphodiester and those in lower case are phosphorothioate.

Antibodies and Chemicals. The following mouse monoclonal antibodies (mAb) were from BD Pharmingen: FITC-conjugated anti-LAMP-1, FITC-conjugated anti-CD63, APC-conjugated anti-HLA-DR, BD-Horizon™ V450-conjugated anti-CD80, APC-conjugated anti-CD86, APC-conjugated anti-BDCA-2, FITC-conjugated anti-CD123 (IL-3R), biotin-conjugated anti-HLA-DR and purified anti-TfR. Purified rabbit mAb anti-EEA1 was from Cell Signaling Technology. Purified rabbit polyclonal anti-LAMP1 antibody was from Abcam. Alexa Fluor (AF)488-, AF568- and AF647-conjugated goat anti-rabbit F(ab’)2 IgG, AF488-conjugated goat anti-mouse F(ab’)2 IgG and APC-conjugated streptavidin were from Molecular Probes (Invitrogen). Live-Dead Fixable Aqua Dead Cell Stain kit was from Invitrogen. Bafilomycin A was from Sigma.

Luciferase assay. HEK293T cells stably transfected with the firefly luciferase gene under the transcriptional control of nuclear factor-κB (NF-κB) together with human TLR7, 8 or 9 (TLR-DST) were made in our laboratory. TLR-DST cells were cultured overnight at 5 x 10^4 cells/well in microclear 96-well plates (Greiner bio-one). Cells were stimulated with serial dilutions of compounds in duplicate for 6 hours and then lysed with cell culture lysis reagent (Promega), according to manufacturer’s
instructions. Luciferase assay substrate (Promega) was added and luciferase activity measured by SpectraMax L microplate reader (Molecular Devices). Luciferase activity was expressed as fold induction over non-stimulated cells.

**Donors.** Buffy coats from healthy HIV-, hepatitis B virus-, and hepatitis C virus-negative donors were obtained from the Blood Transfusion Section, Alta Val D'Elsa Hospital, Poggiobonsi, Italy. Informed consent was obtained before all blood donations. The study protocol was approved by the Novartis Research Center ethical committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

**Purification and stimulation of pDCs.** Peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation, following standard procedures. Highly purified pDCs (\(\geq 98\%\) BDCA-2\(^+\) CD123\(^+\)) were obtained with Diamond Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec), which consists of magnetic depletion of non-pDCs followed by positive selection of pDCs with anti-BDCA-4 antibody. pDCs were cultured at \(5 \times 10^4\) per well in 96-round bottom plates in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (HyClone), glutamine, penicillin and streptomycin. Human rIL-3 (100 ng/ml, R&D Systems) was added to the cultures for 16-24 hours before stimulation with TLR7 agonist small molecules or CpGs to keep pDCs alive\(^32\).

**Cytokine production, CD80 and CD86 up-regulation and compound uptake.** pDCs were stimulated with different concentrations of TLR7 agonist small molecules or CpGs overnight. IFN-\(\alpha\) was quantified with human IFN-\(\alpha\) multi-subtype ELISA kit (PBL Biomedical Laboratories). TNF-\(\alpha\) and IL-6 were measured with the human proinflammatory 7-plex (TNF-\(\alpha\), IL-6, IFN-\(\gamma\), IL-10, IL-
12p70, IL-1β, IL-8) tissue culture kit (MSD Technology). Other cytokines were produced at very low concentrations, except IL-8 that was produced constitutively and poorly induced upon stimulation (data not shown). pDCs were washed twice with ice-cold PBS, stained with anti-CD80 Horizon™ V450 and anti-CD86-APC and analyzed for CD80 and CD86 expression as well as for the fluorescence due to compound uptake by FACS LSRII instrument (Becton Dickinson) using BD FACSDiva™ v6.1.3 software (Becton Dickinson). Data were analyzed using FlowJo software (Treestar Inc.).

**Confocal immunofluorescence microscopy.** pDCs were incubated with fluorescent compounds (TLR7 agonist small molecules or CpG) for 90 minutes. Cells were washed, fixed with 1% paraformaldehyde in PBS at room temperature for 15 minutes and permeabilized with saponin buffer (0.5% saponin, 1% BSA in PBS). Samples were blocked for 30 minutes with Image-iT FX signal enhancer (Invitrogen) and stained with the indicated antibodies in saponin buffer. Cells were cytocentrifuged on glass slides and mounted with SlowFade Gold antifade reagent with 4,6 diamidino-2-phenylindole (DAPI) from Invitrogen), as described. Images were acquired sequentially using separate laser excitations to avoid cross-talks between different fluorophores using either a Nikon Eclipse TE2000, Radiance 2100 Confocal System (Bio-Rad) with the acquisition software Lasersharp 2000 (Zeiss) and processed with Volocity 3.6 (Improvision Inc.), or with a LSM 710 confocal microscope System (Zeiss) and processed with Zen2008 software (Zeiss). An oil-immersion objective (63X, 1.4 numerical aperture, NA), with the pinhole set for a section thickness of 0.8 μM (pinhole set to 1 airy unit in each channel) was used. Unless otherwise stated, the images show one representative cell, as checked by DAPI staining of the nucleus, of at least 30 cells analysed for each sample.
Results

Fluorescently labeled R848, R837 and SM360320 are TLR7 agonists.

Structure-activity relationship (SAR) analysis of R848 led us to the identification of a “tolerant” linker site. We used this site to conjugate R848, R837 and SM360320 (SM) with TAMRA or AF488 fluorophores (Fig. 1). We then assessed the biological activity of these fluorescent molecules on HEK293T cells stably transfected with the luciferase gene under the transcriptional control of NF-κB and TLR7 (TLR7-DST) and compared them with their unlabeled parent molecules. R848 (Fig. 1A) activity on TLR7-DST was strongly decreased upon conjugation with TAMRA (R848-TAMRA EC$_{50}$ 50 μM vs R848 0.5 μM), R837 (Fig. 1B) activity was slightly decreased upon TAMRA conjugation (EC$_{50}$ 65.8 μM vs R837 23.1 μM) and unaltered upon AF488 conjugation (EC$_{50}$ 22.7 μM vs R837 23.1 μM), while SM (Fig. 1C) activity was not affected by conjugation with either fluorophore (SM-TAMRA EC$_{50}$ 0.9 μM, SM-AF488 EC$_{50}$ 0.4 μM vs SM 0.7 μM). All fluorescent compounds were inactive on TLR8-DST (Fig. S1).

The activity of the fluorescently labeled TLR7 agonists was tested on human pDCs that express TLR7 and 9. For practical reasons in this study pDCs were cultured with IL-3 for 18-24 hours before stimulation with TLR7 agonist small molecules or CpG to prevent them from dying. pDCs were stimulated overnight with labeled or unlabeled R848, R837 or SM360320. Cytokine concentrations in the supernatants were measured while cells were stained for CD80 and CD86 and analyzed by flow cytometry for co-stimulatory molecule expression and for compound uptake (TAMRA or AF488 fluorescence). R837-TAMRA and R837-AF488 induced the production of IFN-α, TNF-α and IL-6 and the up-regulation of CD80 and CD86 in a dose-dependent manner (Fig. 2). Internalization of R837-TAMRA and R837-AF488 was readily detected by flow cytometry. Similar experiments were
performed with fluorescent R848 and SM (Fig. S2A-B). All the fluorescent compounds tested induced mainly IFN-α, although production of TNF-α and IL-6 and up-regulation of CD80 and CD86 were also observed, similar to the parent compounds and CpG-B (Fig. S2C). The ability of each fluorescent compound to activate human pDCs with respect to its parent compound was in good agreement with the EC₅₀ measured on TLR7-DST (Fig. 1).

**Imidazoquinolines localize to the MIIC of human pDCs**

The localization of fluorescent TLR7 agonists in pDCs was determined using the protocol applied by Guiducci et al. to assess the localization of CpG-A and CpG-B in these cells. pDCs were incubated with fluorescent TLR7 agonists or CpGs and stained for LAMP-1, CD63 or TfR to identify distinct classes of endosomes. As revealed by confocal microscopy (Fig. 3A-B), both imidazoquinolines labeled with TAMRA co-localized with CpG-B in LAMP-1⁺CD63⁺ endosomes, but not with CpG-A. As a control, the ethanol amine-capped TAMRA fluorophore when used alone did not localize in any intracellular compartment (data not shown). On the other hand, neither R837-TAMRA nor CpG-B-TAMRA localized to TfR⁺ endosomes, while CpG-A-TAMRA showed a partial co-localization with TfR, and almost no co-localization with R837-AF488 (Fig. 3C). The ability of CpGs to induce IFN-α production by freshly isolated human pDCs has been correlated with their localization in TfR⁺ endosomes (early/recycling endosomes). However we found that fluorescently labeled TLR7 agonist small molecules, like their parent compounds and CpG-B, induced pDCs pre-cultured in IL-3 to produce high amounts of IFN-α, yet neither R837-TAMRA nor CpG-B-TAMRA co-localized with TfR. To confirm the absence of localization in early endosomes we analyzed fluorescent TLR7 agonist small molecules and CpG-B for co-localization with EEA1. None of the fluorescent TLR7 agonist small molecules and CpG-B localized to EEA1⁺ endosomes (Fig. 3D and data not shown).
Collectively, these data show that imidazoquinolines do not diffuse randomly inside pDCs but concentrate in LAMP1+CD63+ endosomes despite the fact that they are cell permeable and thus able to spread readily throughout the cell.

LAMP-1 and CD63 are markers of late endosomes and lysosomes, which are characterized by an acidic pH \(^{34}\). Since in pDCs, like in other antigen presenting cells, the endolysosomal compartment is specialized in antigen presentation onto MHC class II molecules (MHC II) \(^{16}\), we stained for HLA-DR, which is a MHC II receptor, and LAMP-1 to check if the endosomes where imidazoquinolines concentrated belonged to the MIIC. As shown in Fig. 4, we found that R848-TAMRA, R837-TAMRA and R837-AF488 localized to endosomes that were positive not only for LAMP-1 but also for HLA-DR. In addition, R837-TAMRA and R837-AF488 co-localized with each other in HLA-DR\(^+\) endosomes. As a control, the ethanol amine-capped AF488 fluorophore alone did not localize in any intracellular compartment (data not shown).

**Purine-like TLR7 agonist SM co-localizes with imidazoquinolines in the MIIC**

To assess if localization in the MIIC is a general feature of TLR7 agonists, we looked at fluorescent SM, a purine-like compound that is structurally distinct from imidazoquinolines. As shown in Figure 5, SM labeled with TAMRA co-localized with HLA-DR and SM-AF488 co-localized with LAMP-1. Moreover, SM-AF488 co-localized with SM-TAMRA and with R837-TAMRA. Thus, TLR7 agonists concentrated in LAMP-1+CD63+HLA-DR\(^+\) endosomes of pDCs independently of the compound chemical scaffold and the fluorophore used for labeling. While we were writing this paper, Shukla et al. described the synthesis of yet another imidazoquinoline conjugated with different fluorophores \(^{35}\). These authors observed an intracellular localization of these compounds in the murine J774 macrophage cell line that, although not characterized further, was compatible with our observations.
Bafilomycin A prevents the concentration of small molecule TLR7 agonists in the MIIC.

The pH of the MIIC is acidic for optimal proteases activity necessary for the generation of peptides to be complex with MHC II molecules \(^{36}\). The acidic luminal environment is maintained by v-ATPase, which pumps protons into the MIIC lumen. To investigate if the acidic pH plays a role in the concentration of TLR7 agonist small molecules in the MIIC we used bafilomycin A (BafA) to block v-ATPase and raise the MIIC pH \(^{31}\). As shown in Figure 6, pre-treatment of pDCs with BafA resulted in the near complete inhibition of SM-AF488 (A) and R837-TAMRA (B) localization in LAMP1⁺HLA-DR⁺ endosomes. By contrast, CpG-B-TAMRA localization in the BafA treated and untreated cells were indistinguishable (C). Moreover, BafA strongly inhibited R837-TAMRA uptake while leaving CpG-B-TAMRA uptake unaltered (D), confirming what we observed by confocal microscopy (B-C).

In line with the fact that BafA is a well-known inhibitor of TLR7 and 9 induced signaling \(^{26}\), in our experiments BafA blocked the production of IFN-α, TNF-α and IL-6 in response to SM-AF488, R837-TAMRA or CpG-B-TAMRA by pDCs (data not shown). Blocking of TLR9 signaling by BafA was likely due to inhibition of proteases responsible for TLR9 cleavage \(^{23,25}\). Although this mechanism could contribute also to the blockade of TLR7 signaling \(^{23,25}\), our findings highlighted that the acidic pH of the MIIC is required for the localization of TLR7 agonist small molecules in there.

To get further insights on the mechanism of entry and accumulation of small molecule TLR7 agonists we studied the effect of BafA on R837-TAMRA uptake over time. As shown in Figure 7A, while BafA had very little effect on the R837-TAMRA uptake at 30 minutes, it blocked further uptake of the compound almost completely at later time points. As expected, CpG-B-TAMRA uptake was almost unaffected by BafA (Fig. 7B). To understand if the internalization of TLR7 agonist small molecules was due to passive diffusion or an active mechanism, we compared R837-TAMRA uptake at 4°C vs 37°C (standard culture condition). While the R837-TAMRA uptake after 30 minutes of
incubation at 4°C was only marginally reduced compared to 37°C, further accumulation of the compound was blocked at 4°C. These results showed that an early passive diffusion of R837 is followed by an active mechanism that dramatically increases the accumulation of the TLR7 agonist in the MIIC (Fig. 7A). No CpG-B-TAMRA internalization occurred at 4°C (Fig. 7B), in agreement with previous data showing that CpG-B enters cells via an active mechanism involving rapid translocation to tubular lysosomal compartment (LAMP-1+) from early endosomes (EEA1+TfR+) 13. Based on these results we propose that R837, which is a cell permeable weak base, passively diffuses inside the cells (fast phase, v-ATPase independent, insensitive to 4°C) and then starts to accumulate in the acidic vesicles of the MIIC because it gets protonated and therefore trapped in there (slow phase, v-ATPase dependent, blocked at 4°C; Fig. 7C) 37.
Discussion

To understand the mechanism of action of small molecule TLR7 agonists more completely, we labeled R848, R837 and SM36020 with TAMRA or AF488 fluorophore and studied their sub-cellular localization. All of the fluorescent compounds retained TLR7 agonist activity and induced human pDCs to produce cytokines and IFN-α and increase expression of co-stimulatory molecules. Examination of pDCs by confocal microscopy revealed that all compounds co-localized with CpG-B in LAMP-1⁺CD63⁺HLA-DR⁺ endosomes, independently of their chemotype or labeling fluorophore. Our observations are in line with a very elegant study by Sadaka et al. showing that MHC II molecules converge with LAMP-1 and CD63 (but not with TfR and EEA1 markers) in human pDCs treated with IL-3. LAMP-1 and CD63 are markers of late endosomes and lysosomes while HLA-DR identifies MHC II molecules. Therefore, the vesicles where small molecule TLR7 agonists accumulate appear to belong to the MIIC. Indeed, in pDCs, like in other antigen presenting cells, the endolysosomal compartment is specialized for MHC II antigen presentation and is considered as a lysosome-related organelle. The function of MHC II molecules is to access the endolysosomal network, bind peptides derived from the antigens and display them on the cell surface to CD4⁺ T cells. Notably, the regulation of MHC II expression and transport is completely different in pDCs compared to conventional DCs (cDCs) from both humans and mice. In particular, cDCs halt the synthesis and degradation of MHC II after activation, whereas pDCs maintain synthesis and turnover of MHC II. As a result pDCs are less efficient at presenting antigens than cDCs but their more dynamic mode of antigen presentation should be advantageous in counteracting viruses that exhibit mutation rates. Peptide binding by MHC II molecules requires the degradation of the invariant chain (Ii) by endolysosomal proteases. Recently, these proteases have been implicated in the activation of TLR7 and 9. Our data showing that small molecule TLR7 agonists and CpG-B localize to MIIC suggest that this compartment is the

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cross-road where antigens and MHC II molecules are assembled and nucleic acid sensing TLRs signal upon encounter with their ligands. The commonality of proteolytic cleavage to form functionally relevant forms of both MHC II and TLRs and their co-localization could impact the way pDCs inform T cells as to the specific nature of distinct pathogenic threats.

Small molecule TLR7 agonists and CpG-B did not localize to early endosomes (TfR$^+$ or EEA1$^+$), yet they induced high amounts of IFN-α production. These results are in contrast with the proposed model that in human pDCs TLR9 signaling from early endosomes (TfR$^+$) leads to IFN-α production while signaling from late endosomes (LAMP-1$^+$) leads to upregulation of co-stimulatory molecules$^{28}$. However, our observations are in line with a recent report from Sasai et al. showing that in mouse bone marrow derived macrophages TLR9 signals leading to IFN-I production are generated in acidic endosomes (LAMP-2$^+$LysoTracker$^+$)$^{42}$. It is unclear why CpG-B is a poor inducer of IFN-α by freshly isolated pDCs$^{28}$ but a good inducer for IL-3 pre-treated pDCs$^{43}$. In both pDC populations CpG-B localizes to LAMP-1$^+$ endosomes suggesting that IL-3 treatment leads to changes in the constituents of these vesicles. Indeed, IL-3 treatment induced the re-localization of MHC II to LAMP-1$^+$ endosomes and it is tempting to speculate that key IFN-I signaling components, such as interferon regulatory factor 7, are recruited to the MIIC in response to IL-3 treatment.

A key feature of the MIIC is low pH. Blockade of vesicle acidification by BafA or NH$_4$Cl (data not shown) abolished the accumulation of R837-TAMRA in the MIIC suggesting that the electrochemical proton gradient is the driving force for the accumulation of small molecule TLR7 agonists in this compartment. Interestingly, a similar mechanism has been shown to cause the accumulation of neurotransmitters and hormones into secretory vesicles$^{44}$. At the acidic pH found in the MIIC of pDCs (pH 5-6$^{28}$), small molecule TLR7 agonists are predicted to be positively charged and protonated and are expected to reach concentrations in the acidic compartment ~100 fold higher than the rest of the cell$^{37}$. This substantial accumulation of compound is consistent with what we
observed in live pDCs (data not shown) and could serve to drive low affinity interactions of small molecule TLR7 agonists with TLR7 and/or a co-receptor molecule above the threshold necessary for signaling.
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Authorship

**Contribution:** C.R. performed experiments, analyzed data and edited the manuscript; I.C.-T. and J.R. supervised the synthesis of small molecule TLR7 agonists; L.G.-S. performed experiments; E.H. and Y.I. synthesized small molecule TLR7 agonists; S.T., C.S. and S.N. advised on flow cytometry experiments and analysis; L.M. advised on study design; J.T. designed and supervised the synthesis of small molecule TLR7 agonists; N.M.V. and E.D.G. advised on study design and wrote the paper; E.S. designed, supervised and performed experiments and wrote the manuscript.

**Conflict-of-interest disclosure:** All authors are employees of Novartis.

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References


**Figure legends**

**Figure 1. Fluorescently labeled R848, R837 and SM360320 are TLR7 agonists.** R848 (A), R837 (B) and SM (C) were conjugated with TAMRA (in red) or with AlexaFluor488 (AF488, in green) fluorophores at the indicated tolerant linker site. A spacer was introduced together with TAMRA (in orange). Both fluorophores were a mixture of 5-6 regioisomers. Structures depicted here represent the 5-regioisomer. The biological activity of these fluorescently-labeled molecules was assessed on HEK293T cells stably transfected with the luciferase gene under the transcriptional control of NFκB and TLR7 (TLR7-DST) in parallel with their non fluorescent parent molecules. Luciferase activity was expressed as fold induction (FI) over non stimulated cells. The effective concentrations required to induce half of the maximum response (EC$_{50}$) are reported.

**Figure 2. Fluorescently labeled R837 activates human pDCs.** Human pDCs, purified from fresh buffy coats and pre-cultured with IL-3 for 24 hours, were stimulated with different concentrations of R837, labeled or not with TAMRA or AF488. After overnight incubation, cytokine concentrations in the culture supernatants were assayed while cells were analyzed by flow cytometry for CD80 and CD86 expression and for compound uptake (TAMRA or AF488 fluorescence). Data shown are from one donor and are representative of at least three independent donors.
Figure 3. Imidazoquinolines localize to LAMP1⁺CD63⁺ endosomes but not to TfR⁺EEA-1⁺ endosomes in pDCs. Human pDCs, pre-cultured overnight with IL-3, were stimulated for 90 minutes with: (A) R848-TAMRA (20 µM), R837-TAMRA (50 µM), CpG-B-TAMRA (10 µM) or CpG-A-TAMRA (10 µM); (B) R837-TAMRA (50 µM), CpG-B-TAMRA (10 µM), CpG-A-TAMRA (10 µM) or R837-TAMRA (50 µM) together with CpG-B-FITC (10 µM); (C) R837-TAMRA (50 µM), CpG-B-TAMRA (10 µM), CpG-A-TAMRA (10 µM) alone or together with R837-AF488 (20 µM); (D) R837-AF488 (20 µM), CpG-B-FITC (10 µM), R848-TAMRA (20 µM) or CpG-B-TAMRA (10 µM). Cells were fixed, permeabilized and stained with anti-LAMP-1-FITC antibody (A), anti-CD63-FITC antibody (B), purified mouse mAb anti-TfR followed by anti-mouse antibody labeled with AF488 (C) or purified rabbit mAb anti-EEA1 followed by anti-rabbit antibody labeled with AF568 (D, top and upper middle panels) or AF488 (D, lower middle and bottom panels). Images were acquired using a Biorad TE2000-U (A-B) or a ZEISS LSM 710 (C-D) confocal microscope and an oil-immersion objective (63X, 1.4 NA), with the pinhole set for a section thickness of 0.8 μM (pinhole set to 1 airy unit in each channel). Images were acquired sequentially using separate laser excitations to avoid cross-talks between different fluorophores and processed with Zen2008 software. Images show single cells from one representative donor out of three.

Figure 4. Fluorescently labeled R848, R837 and SM360320 localize to LAMP1⁺HLA-DR⁺ endosomes in pDCs. pDCs, pre-cultured overnight with IL-3, were stimulated with R848-TAMRA (20 µM), R837-TAMRA (50 µM), R837-AF488 (20 µM) alone or together with R837-TAMRA (50 µM) for 90 minutes. Cells were fixed, permeabilized, stained with purified rabbit polyclonal anti-LAMP1 antibody followed by anti-rabbit antibody labeled with AF488 (top and upper middle) or AF568 (lower middle) and anti-HLA-DR-APC. Images were acquired using a ZEISS LSM 710 confocal microscope and an oil-immersion objective (63X, 1.4 NA), with the pinhole set for a section thickness of 0.8 µM.
(pinhole set to 1 airy unit in each channel). Images were acquired sequentially using separate laser excitations to avoid cross-talks between different fluorophores and processed with Zen2008 software. Images show single cells from one representative donor out of three.

**Figure 5. Purine-like TLR7 agonist SM360320 co-localizes with imiquimod in the MIIC.** pDCs, pre-cultured overnight with IL-3, were stimulated with SM-TAMRA (20 µM) or with SM-AF488 (3 µM), alone or together with SM-TAMRA (20 µM) or R837-TAMRA (20 µM), for 90 minutes. Cells were fixed, permeabilized, stained with anti-HLA-DR-APC or purified rabbit polyclonal anti-LAMP1 antibody followed by anti-rabbit antibody labeled with AF647. Images were acquired using a ZEISS LSM 710 confocal microscope and an oil-immersion objective (63X, 1.4 NA), with the pinhole set for a section thickness of 0.8 µM (pinhole set to 1 airy unit in each channel). Images were acquired sequentially using separate laser excitations to avoid cross-talks between different fluorophores and processed with Zen2008 software. Images show single cells from one representative donor out of three.

**Figure 6. BafA prevents the localization of small molecule TLR7 agonists to the MIIC.** pDCs, pre-cultured overnight with IL-3, were treated with bafilomycin A (BafA, 100 nM) for 2 hours before stimulation with SM-AF488 at 3 µM (A), R837-TAMRA at 50 µM (B) or CpG-B-TAMRA at 10 µM (C). After 90 minutes of stimulation, cells were fixed, permeabilized, stained with anti-HLA-DR-APC and purified rabbit polyclonal anti-LAMP-1 antibody followed by anti-rabbit antibody labeled with AF568 (A) or with AF488 (B-C). Images were acquired using a ZEISS LSM 710 confocal microscope and an oil-immersion objective (63X, 1.4 NA), with the pinhole set for a section thickness of 0.8 µM (pinhole set to 1 airy unit in each channel). Images were acquired sequentially using separate laser excitations to avoid cross-talks between different fluorophores and processed with Zen2008 software. Images show several cells. (D) After 18 hours of stimulation with R837-TAMRA (50 µM) or CpG-B-
TAMRA (10 µM), compound uptake by pDCs was evaluated by flow cytometry. Images show several cells from one representative donor out of three. Cells in squares are shown in higher magnification on the right.

**Figure 7. Bafilomycin A prevents the concentration of imiquimod in the MIIC.** pDCs, pre-cultured with IL-3 for 24 hours, were treated or not with BafA (100 nM) for 2 hours. Then, cells were stimulated with R837-TAMRA at 50 µM (A) or CpG-B-TAMRA at 10 µM (B) for the indicated times at 37°C or at 4°C. Cells were stained with Live/Dead and analyzed by flow cytometry for compound uptake gating on live cells. The mean fluorescence of cells alone cultured under the same conditions was subtracted (delta mean fluorescence). Cell viability was not affected by BafA treatment. Data were obtained with pDCs purified from a single donor and were reproduced with pDCs from two other donors. (C) Model for the concentration of TLR7 agonist small molecules in acidic endosomes based on the previous data. R837 is a cell-permeable weak base that passively diffuses everywhere inside the cell (fast phase, v-ATPase-independent) and then starts to accumulate in the class II loading compartment where it gets protonated and therefore trapped (slow phase, v-ATPase-dependent). In the presence of BafA, v-ATPase is blocked and endosomal pH is no longer acidic. Therefore, R837 does not get protonated in endosomes and does not accumulate there.
Fig. 1

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**Fig. 3**

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**fig. 7**

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Small molecule Toll-like receptor 7 agonists localize to the MHC class II loading compartment of human plasmacytoid dendritic cells

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