Peptides conjugated to 2-alkoxy-8-oxo-adenine as potential synthetic vaccines triggering TLR7

Geoffroy P.P. Gential[a,1], Tim P. Hogervorst[a,1], Elena Tondini[b], Michel J. van de Graaff[a], Herman S. Overkleeft[a], Jeroen D.C. Codée[a], Gijsbert A. van der Marel[a], Ferry Ossendorp[b], Dmitri V. Filippov[a,*]

[a] Leiden Institute of Chemistry and The Institute for Chemical Immunology, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands
[b] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands

A B S T R A C T

Covalent linking of immunogenic oligopeptides with synthetic Toll-like receptor ligands is a useful approach to develop self-adjuvanting vaccines. In particular, small-molecule based agonists of Toll-like receptor 7 (TLR7) that are derived from 8-oxo-adenine core are potentially promising because these chemically robust TLR7 ligands can be connected to peptide T-cell epitopes via straightforward solid-phase peptide synthesis. In this contribution we present the synthesis of a Boc-protected 9-benzyl-2-alkoxy-8-oxo-adenine building block and its application in the online solid phase synthesis of three peptide conjugates that differ in the position of the TLR7 ligand within the peptide. The conjugates are able to induce dendritic cell maturation and T cell proliferation while the position of the ligand impacts T cell proliferation potency.

Toll-like receptors (TLRs) are part of the mammalian innate immune system that forms the first line of defence against pathogens by recognising pathogen associated molecular patterns (PAMPs).[1] Upon recognition of a specific PAMP by the corresponding TLR, a signal transduction pathway is started that activates the immune system. Ten different human TLRs can be discerned that are situated either at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in intracellular compartments (TLR3, TLR7, TLR8 and TLR9).[2] Each TLR recognizes PAMPs of certain structural identity and intracellular TLRs bind to nucleic acids of various origin. TLR8 binds to viral and bacterial RNA.[3,4] TLR9 recognizes bacterial and viral single stranded DNA, the latter is also recognized by TLR7.[5,6] As modulators of the immune system TLR ligands are important drug targets and much research has been directed to the development of small-molecule TLR agonists.[7] A prime example of a TLR7 agonist is imiquimod[8] that is used for topical treatment of a variety of skin diseases.[9,10] In the last decade structure–activity studies on small molecules have resulted in specific and non-specific agonists for TLR7 and TLR8. Well known examples are represented by imidazoquinolines (e.g. imiquimod) and 8-oxo-adenine derivatives.[11,12] These agonists, either as such or conjugated to other molecular entities have been explored as adjuvants in the framework of the development of new vaccines and immunotherapies.[11,13,14] For

example, to improve the potency and/or to prevent toxic side effects TLR7 ligands are conjugated to macromolecules such as phospholipids, polysaccharides and peptides.[15,16] One approach towards the development of fully synthetic vaccine modalities is directed to the design, synthesis and evaluation of conjugates, comprising a structurally defined TLR agonist, covalently connected to an oligopeptide epitope.

Several of these conjugates exhibit improved immunological properties in comparison with a mixture of the composing components.[18–20] Such conjugates in which a TLR7 ligand is covalently connected to an antigenic peptide have been prepared as well. We synthesized azide-containing analogues **1b-g** of the TLR7 ligand 9-benzyl-8-oxo-2-butoxyadenine **1a**, that can be applied in the synthesis of conjugates (Fig. 1).[17,21] For instance, copper(I) catalysed cycloaddition of **1b** to the appropriate alkynyl-peptide gave conjugate **2** featuring – besides the TLR7 ligand – the murine cytotoxic T lymphocyte epitope, SIINFEKL.[17] Although this conjugate give rise to enhanced antigen presentation in vitro, it unexpectedly lack the ability to induce maturation of dendritic cells (DC). The latter action is crucial for the induction of T-cell mediated immunity. The position in the TLR7 agonist to which the peptide epitope is connected proved to be of prime importance for the immunological activity of the resulting conjugate: Jin and co-workers revealed that attachment of the 8-hydroxyadenine based TLR7 agonist

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1a R = CH$_3$CH$_2$, n = 1
1b R = N$_3$(CH$_2$CH$_2$O)$_2$, n = 1
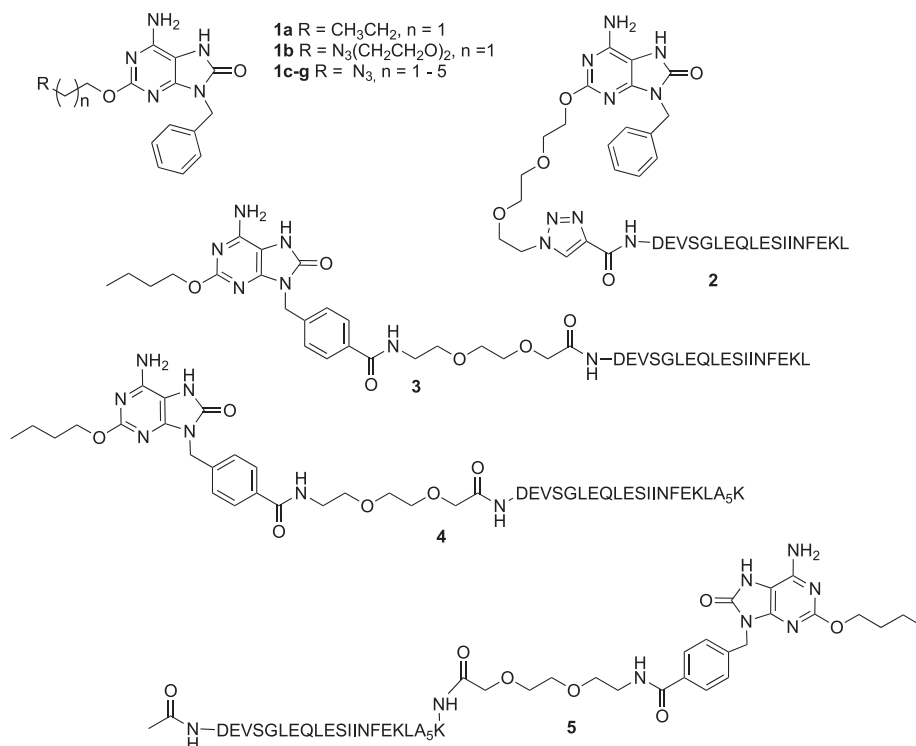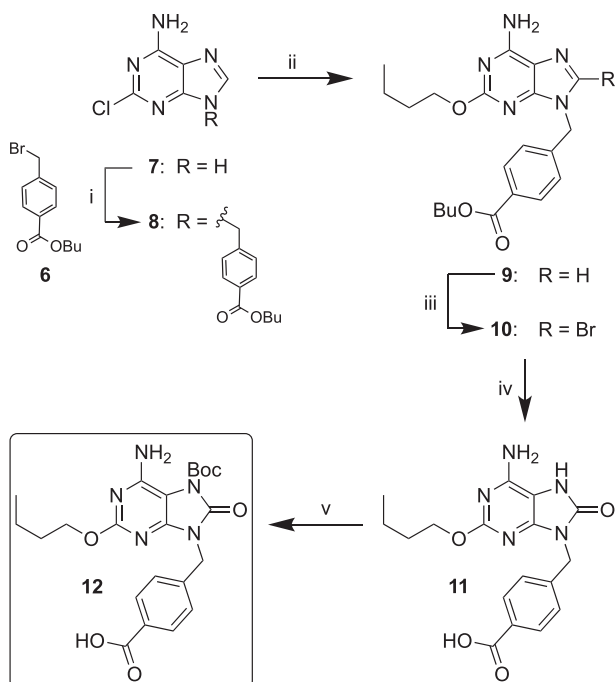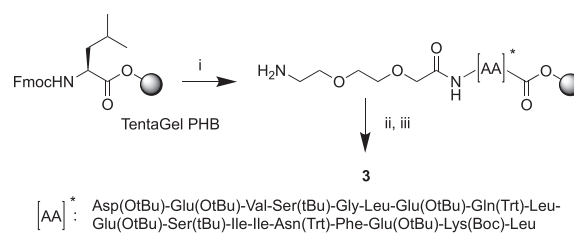**1c-g** R = N$_3$, n = 1 - 5

Fig. 1. TLR7 ligand (**1**) and its conjugate (**2**) synthesized previously[17] by us and TLR7 conjugates **3**, **4** and **5** that are subject of the here-presented studies.

Scheme 1. Synthesis of compound 12. *Reagent and conditions:* i) 1 M TBAF in THF, RT, 92% ii) 1) NaH, *n*-BuOH, 120 °C 2) H$_2$SO$_4$, 80 °C, 71% iii) Br$_2$, NaOAc, DCM, 94% iv) NaOH, H$_2$O, MeOH, reflux, 89% v) NaOH, Boc$_2$O, H$_2$O, dioxane, 35%.

[AA]*: Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu

Scheme 2. Synthesis of conjugate 3. *Reagents and conditions:* i) standard SPPS synthesis ii) **12**, HCTU, Dipea, NMP iii) TFA/H$_2$O/TIS (95/2.5/2.5).

of OVA-derived peptide DEVSGLEQLESIINFEKL to give conjugate **3**. The same TLR7 ligand was installed at both the *N*-terminus and at the side chain of the C-terminal lysine residue in DEVSGLEQLESIINFE-KLA$_5$K to give conjugates **4** and **5**, respectively. The A$_5$K extension on C-terminus of the peptide is introduced to allow a comparison of the immunological activity of the conjugates having the TLR7 agonist installed at either the *N*-terminus or close to the C-terminus of the epitope containing peptide. Such design of the conjugates was used by us in the previous studies and it was demonstrated that the presentation of the CTL-epitope was not precluded.[17] An ethylene glycol spacer, exhibiting minimal steric hindrance is selected for its favourable influence on the biological activity of the TLR7 ligand upon conjugation, as was shown in previous studies.[16]

The assembly of the projected conjugates (**3–5**) was performed with the aid of an automated synthesizer using a solid phase peptide synthesis (SPPS) protocol. To achieve this, the previously reported carboxyl functionalized TLR7 ligand **11** (Scheme 1) was prepared by adaption of published procedures.[11,17,21,23] However, in our hands this building block proved to be unsuitable for SPPS due to its poor solubility as previous described.[16] A major improvement was achieved by protection of the purine base by a Boc group to give soluble building block **12** that could be used successfully in a SPPS setting (Scheme 1). In the first step of the synthetic route to **12** commercially available 2-chloroadenine **7** was selectively benzylated at the N-9 position using alkylating reagent **6** (Scheme 1). This reagent was prepared by acid

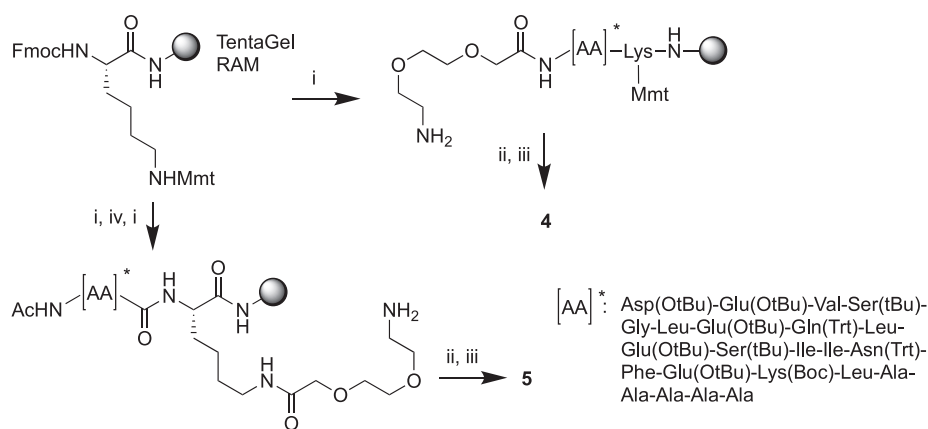via its 9-benzyl moiety to an oligopeptide did mediate DC activation.[15]

Here we describe the synthesis of three conjugates (Fig. 1, **3–5**), in which the well-established TLR7 ligand 9-benzyl-8-oxo-2-butoxy-adenine[11,22] is covalently connected through its 9-benzyl moiety to the CTL (SIINFEKL) epitope. A derivative of 9-benzyl-8-oxo-2-butoxy-adenine having a carboxyl function at the para position of the benzyl moiety was used for the covalent attachment of the TLR7 ligand to the *N*-terminus

Scheme 3. Synthesis of conjugates **4** and **5**. *Reagents and conditions:* i) standard SPPS synthesis; ii) **12**, HCTU, DIPEA, NMP; iii) TFA/H$_2$O/TIS (95/2.5/2.5); iv) 1% TFA, DCM.
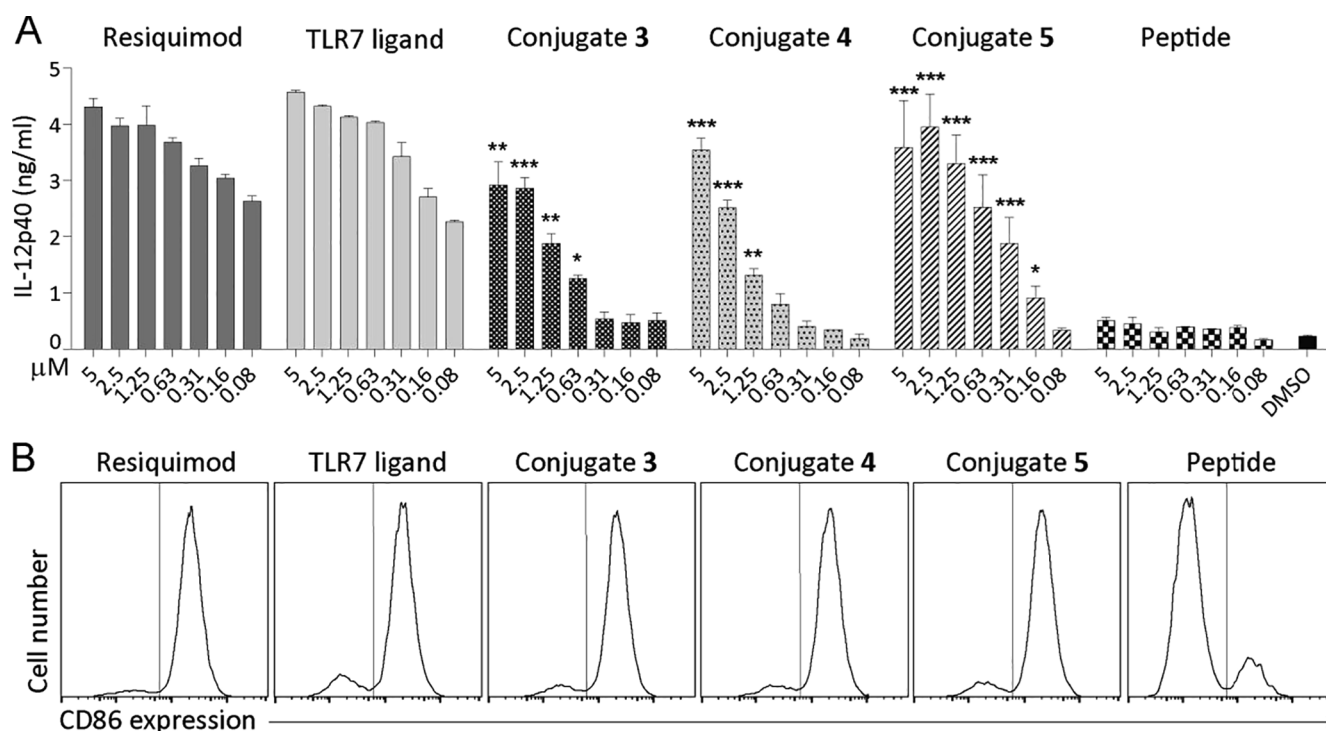

Fig. 2. Induction of dendritic cell maturation by TLR7 conjugates was measured by IL-12p40 cytokine production (A) and expression of the costimulatory molecule CD86 (B), at 5 μM. Resiquimod is a marketed TLR7/8 agonist and was used here as comparison to the oxoadenine TLR7 ligand used in conjugates **3–5**. For IL-12p40, means of two independent experiments ± SEM are reported. Statistical significance was determined for the conjugates versus peptide at the same concentration by one-way ANOVA, followed by multiple comparison with Bonferroni correction. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

mediated condensation of p-toluic acid and *n*-butanol, followed by radical bromination of the obtained ester, using *N*-bromosuccinimide and AIBN (See Supporting Information). It was established that benzylation of 2-chloroadenine **7** with bromide **6** using 1 M TBAF in THF outperformed procedures featuring bases other than TBAF. For instance, both selectivity and yield dropped when K$_2$CO$_3$ was used as a base. The quality of the TBAF/THF solution, most likely its water content, influenced the yield of benzylation of the N-9 position of the adenine derivative.[17,21] Nucleophilic aromatic substitution of 2-chloroadenine derivative **8** with sodium *n*-butoxide at 120 °C was accompanied by partial hydrolysis of the butyl ester. The butyl ester was recovered by acidifying the crude reaction mixture with H$_2$SO$_4$ and heating it at 80 °C for 2 h to give adenine derivative **9**. Electrophilic aromatic substitution of **9**, using elementary bromine under mild basic conditions gave **10** in high yield. Subsequent direct hydrolysis of the bromine at the C-8 position in **10** with aqueous NaOH was unsuccessful, however, when **10**

was refluxed for three days in a mixture of water and methanol the product **11** could be obtained. Compound **11**, which appeared to be insoluble in most solvents, was isolated by precipitation from aqueous acidic solution followed by collection of the sticky precipitate by centrifugation, washing the precipitate with dilute hydrochloric acid and drying. The impracticability of **11** forced us to consider protection of the purine base in **11** with a SPPS compatible protecting group. Due to the poor solubility of **11** introduction of Boc or trityl protections proved to be cumbersome. Firstly, we tried 2-(Boc-oxyimino)-2-phenylacetonitril (Boc-ON) as the reagent to introduce a Boc protection to the oxoadenine core but this reaction proceeded sluggishly and required long reaction times. After five days of stirring at room temperature with a ten-fold excess of the reagent, LC-MS analysis indicated approximately 50% conversion and after purification **12** was obtained in only 10% yield. This low isolated yield is probably due to an insufficient solubility of **12** in dichloromethane that has been used to recover the product
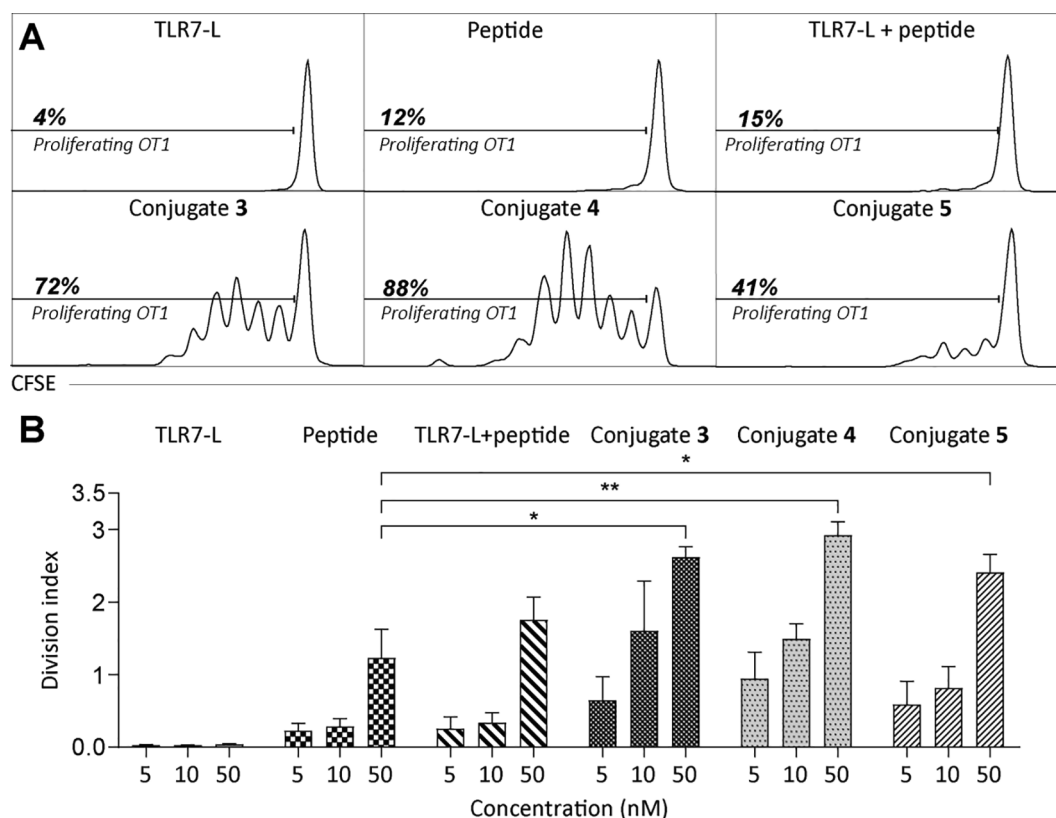
Fig. 3. Antigen processing and presentation of the conjugates by dendritic cells was measured by proliferation of SIINFEKL-specific OT1 T cells. OT1 cells were intracellularly labelled with the CFSE fluorophore and cellular division was measured as cumulative dilutions of CFSE signal. A) Representative proliferation profile of one experiment out of three performed, displayed for 10 nM concentration of each compound. B) Mean division index of OT1 cells upon co-culture with dendritic cells incubated with 5, 10 and 50 nM of the indicated compounds. Significance was determined by one-way ANOVA followed by multiple comparison with Duvett correction. $^*p < 0.05$, $^{**}p < 0.01$.

after work up with aqueous acid (10% $KHSO_4$). A major improvement was achieved by dissolving **11** in an aqueous NaOH solution followed by dropwise addition of di-*tert*-butyl dicarbonate (Boc_2O), subsequent neutralisation of the reaction with HCl allowed the removal of the excess reagent and finally Soxhlet extraction gave **12** (30–40%) and regenerated starting material **11** (~50%). Although the yield of this final protection was somewhat low, the fact that starting material **11** could be regenerated made this approach practical and allowed preparation of **12** in gram quantities. The improved solubility of **12** over **11** in organic solvents made **12** the preferred building block for the forthcoming SPPS.

Having the functionalized TLR7 ligand **12** available, SPPS of conjugate **3** was performed starting from Tentagel® S PHB resin, and using Fmoc chemistry with HCTU as the condensing agent and commercially available protected amino acid building blocks (Scheme 2). After completion of the DEVSGLEQLESIINFEKL sequence, the Fmoc group at the *N*-terminus of the immobilized oligopeptide was removed and Fmoc-protected aminodiethyleneglycol linker was introduced and deprotected using the same conditions as used for the standard Fmoc amino acids. In the final step Boc-protected TLR7 ligand **12** was coupled to the unmasked amine using HCTU as the condensing agent. Finally, removal of all protecting groups and cleavage from the resin was achieved with a TFA to give after RP HPLC purification target conjugate **3** in a yield of 10%. Conjugates **4** and **5** (see Figure 1) were prepared and purified using the same SPPS conditions as described for conjugate **3** but starting with Tentagel® S RAM amide resin, to which Fmoc-Lys(Mmt)-OH was coupled as the first amino acid (Scheme 3). For conjugate **5** the *N*-terminus was acetylated after completion of the DEVS-GLEQLESIINFEKLA$_5$K sequence, Mmt cleavage and elongation of the lysine side chain by introduction of the glycol linker followed by

introduction of Boc protected TLR7 ligand **12**. Removal of all protection groups and cleavage from the resin followed by purification gave conjugates **4** and **5** in 12% and 10% yield respectively. Next, the conjugates were evaluated for their ability to induce dendritic cell maturation and T-cell proliferation. Conjugation at the *para*-position of the benzyl moiety of the TLR7 ligand preserved its ability to promote dendritic cell maturation, as demonstrated by both production of IL-12p40 (Fig. 2A) and a strong upregulation of membrane expression of the costimulatory molecule CD86 (Fig. 2B). All conjugates were functional although their activity was slightly lower compared to the free ligand. Both *N*-terminal conjugates **3** and **4** showed activity down to a concentration of 600 nM. In comparison, the C-terminal conjugate (**5**) displays higher activity down to 160 nM concentration. A crucial step for induction of T cell immunity is represented by uptake and correct processing of the peptide moiety by dendritic cells. This step should lead to epitope presentation (here: the SIINFEKL epitope) by surface MHC molecules to specific T cells. The ability of the conjugates to induce T cell proliferation was tested in vitro with SIINFEKL-specific OT1 T cells. Dendritic cells were pulsed with the conjugates or the unconjugated components and exposed to CFSE-labelled OT1. After three days, OT1 proliferation was measured as CFSE dilution in individually divided cells (Fig. 3 A). The three conjugates all induced significantly enhanced T cell proliferation when compared to unconjugated peptide or a mix of TLR7 ligand and peptide (Fig. 3B). In comparison, the conjugates with the ligand at the *N*-terminal position (conjugates **3** and **4**) performed better than the C-terminal conjugate (conjugate **5**) as shown by the mean division index (Fig. 3B).

To conclude, we here describe that the TLR 7 ligand 9-benzyl-8-hydroxy-2-butoxy-adenine functionalized with a carboxyl group at the para position of the benzyl moiety (i.e. **11**) was made suitable for SPPS

by protection of **11** with the Boc group to give building block **12**. We exemplified this by the preparation of the peptide conjugates **3**, **4** and **5** that contain the TLR-7 ligand attached to different positions within the peptide sequence. Biological evaluation of these conjugates showed that the prepared conjugates are functional and that the position of the ligand influences immunogenicity. We observed induction of dendritic cell maturation upon conjugation both at the N- and C-terminal position, indicating TLR signalling. Specifically, the C-terminal conjugate displayed a higher activity at lower concentrations. This could be explained by differential accessibility of the ligand to the TLR7 receptor depending either on the physical position of the ligand on the peptide or its interaction with the adjacent amino acids. Secondly, we show that all conjugates display proper epitope processing and enhanced antigen presentation, suggesting that conjugation of the ligand allows proteasome-mediated cleavage. However, the two *N*-terminal conjugates induced higher T cell proliferation compared to the C-terminal conjugate. As C-terminal hydrolytic cleavage by the proteasome is crucial for efficient T cell epitope generation[24], the TLR7- ligand at the C-terminal position of the peptide may interfere with the proteolytic process. As efficient antigen presentation is fundamental for effective induction of T cell responses, we argue that *N*-terminal conjugation may be preferred for future development of covalent peptide-based vaccines.

Notes

The authors declare no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.03.048.

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