

IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses

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Abstract

IC31, the combination of a novel immunostimulatory oligodeoxynucleotide containing deoxy-Inosine/deoxy-Cytosine (ODN1a) and the antimicrobial peptide KLKL₅KLK, represents a promising novel adjuvant signaling via the TLR9/MyD88-dependent pathway of the innate immune system. In mice, IC31 induces potent peptide-specific type 1 cellular immune responses, as well as mainly type 1 dominated protein-specific cellular and humoral immune responses. In addition, cytotoxic T lymphocytes were induced, able to kill efficiently target cells in vivo. Activation of murine dendritic cells by IC31 induced efficiently proliferation of naïve CD4⁺ TCR transgenic T cells (DO.11.10) as well as their differentiation into IFN- γ - and IL-4-producing T cells in vitro.

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1. Introduction

The defense against pathogens involves early recognition by the innate immune system followed by an improved adaptive immune response specific for a given antigen [1]. Immunization is a tool that enables the immune system to successfully eliminate pathogens [2]. Traditionally, live attenuated and inactivated pathogens are used for immunization. For obvious reasons recombinant proteins or peptides derived from an antigen are preferable to whole pathogens for vaccinations. However, administered alone in most cases these antigens are weakly immunogenic by themselves. Adjuvants are able to improve the quantity and quality of innate immune

responses by enhancing their speed and duration, and by inducing adequate adaptive immunity [1–3]. Currently, only two vaccine adjuvants are approved universally for human use: aluminum salts and MF59 [6]. Many other adjuvants (e.g. virosomes and exotoxins) are either restrictively approved in some countries, or being currently evaluated in clinical trials. The failure of aluminum salts to stimulate CTL responses and to improve subunit vaccines led to the development of further adjuvants.

Recently, synthetic oligodeoxynucleotides containing CpG-motifs (CpG-ODNs) were described to act as cell mediated immune response inducing adjuvant [7–9]. However, some of these CpG-ODNs may have potential side effects since they induce systemic production of pro-inflammatory cytokines [10,11]. In addition, their nuclease-resistant phosphorothioate backbone is described to have unspecific immunostimulatory activity [12]. We have shown recently that the combination of the cationic polyamino acid poly-L-arginine, which is already immunostimulatory by itself [13–15], with a CpG-ODN results in a potent adjuvant

Abbreviations: BMDC, bone marrow-derived dendritic cell(s); Cy5, cyanin 5; DC, dendritic cell(s); FL, fluorescein-labeled; hmDC, human myeloid dendritic cell(s); MyD88, Myeloid differentiation factor 88; ODN, oligodeoxynucleotide; TLR, Toll-like receptor(s); mTRP-2, murine tyrosinase-related protein 2

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that induces prolonged antigen-specific cellular immune responses in mice [16]. In addition, the CpG-ODN-induced systemic release of pro-inflammatory cytokines was prevented in the presence of poly-L-arginine.

Considering that a combination of two immunomodulatory compounds represents a more potent adjuvant than either alone, we further developed this concept. Cationic antimicrobial peptides are a class of immunostimulatory, positively charged molecules that are able to facilitate and/or improve adaptive immune responses [7]. In particular, immunopotentiating properties have been reported for the synthetic positively charged peptide KLKL₅KLK, which induces potent protein-specific type-2 driven adaptive immunity after prime-boost immunizations [32].

Negatively charged immunostimulatory nucleic acids containing CpG motifs [17,18] or double-stranded RNA, such as poly(I:C) [9,19], represent another class of adjuvants. Immunostimulatory CpG-ODN and poly(I:C) are ligands of the Toll-like receptors TLR9 and TLR3, respectively, which belong to a family of innate receptors specific for pathogen-associated molecular patterns [4,5]. Signaling via some TLR receptors, like TLR9, employs the adaptor protein MyD88 that induces NF- κ B activation, thereby triggering the initiation of immune responses [20]. Initial observations led us to develop oligonucleotides based on poly(I:C): the novel single stranded oligodeoxynucleotide ODN1a was designed to consist of dimeric repeats of deoxy-Inosine/deoxy-Cytosine linked by an unmodified phosphodiester backbone.

Herein, the immunostimulatory characteristics of the combination of ODN1a and KLKL₅KLK, termed IC31, are described. Murine *in vivo* studies show synergistic effects of these compounds on the induction of cellular and humoral immune responses involving the TLR9/MyD88 signaling pathway. Further *in vitro* studies in the murine system indicate that the immunostimulatory effect of IC31 on bone marrow-derived dendritic cells (BMDC) enables the induction of proliferation and differentiation of naïve T cells.

In summary, IC31 shows promise to possess the requirements as an adjuvant for application in humans where strong antigen-specific cellular and humoral immune responses without induction of undesirable side effects are required.

2. Materials and methods

2.1. Mice

C57BL/6 mice (H-2^b) were purchased from Harlan Winkelmann (Borchen, Germany). TLR9 knock-out and MyD88 knock-out mice (both H-2^b genetic background), kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan), and DO11.10 from The Jackson Laboratory (Bar Harbor, ME, USA) were bred in Intercell's own animal facility. Female mice were used for experiments at 6–10 weeks of age. All experiments were carried out according to Austrian Law (Tierversuchsgesetz BGBl Nr. 501/1989).

2.2. Antigens

Crystallized chicken OVA (grade V, Sigma–Aldrich, Austria) was dissolved in endotoxin-free water (Mayrhofer Pharmazeutika, Linz, Austria) at 10 mg/ml and stored at –20 °C. Peptide antigens were OVA_{257–264} (SIINFEKL, H2-K^b, derived from chicken ovalbumin; Bachem, St. Helens, UK), OVA_{265–280} (TEWTSSNVMEERKIKV, I-A^b, derived from chicken ovalbumin; in house synthesis) and mTRP-2_{181–188} (VYDFFVWL, H2-K^b, derived from murine tyrosinase related protein-2; Multiple Peptide Systems, San Diego, CA, USA). Peptides were synthesized in-house by solid phase synthesis on preloaded 2-chlorotriyl resins (Pepchem, Tübingen, Germany) with an ABI 433A (PE Biosystems, Weiterstadt, Germany) or a multiple peptide synthesizer (Syro II, MultisynTech, Witten, Germany). After synthesis, peptides were cleaved from the resin and purified by RP-HPLC. The identity and purity was confirmed by mass spectroscopy using a MALDI-TOF mass spectrometer Reflex III (Bruker, Leipzig, Germany). All peptides were dissolved in DMSO (Merck, Austria) at 10 mg/ml and stored at –20 °C.

OVA_{257–264} peptide was fluorescein-labeled (Fl) while still attached to the resin. In short, an eight-fold molar excess of fluorescein (NHS-ester, Molecular Probes, Eugene, OR, USA) in DMF was added to the resin and the mixture was incubated at room temperature overnight. Unreacted dye was removed by several washes with DMF. The Fl-peptide was cleaved off the resin and purified by HPLC.

2.3. Adjuvants

KLKL₅KLK (NH₂-KLKLLLLLKLK-COOH, purchased from Multiple Peptide Systems, San Diego, CA, USA or synthesized at Intercell for fluorescent labeling) and ODN1a (phosphodiester backbone ODN, 5'- ICI CIC ICI CIC ICI CIC ICI CIC IC-3' from Purimex, Göttingen, Germany) were dissolved in endotoxin-free water (Mayrhofer Pharmazeutika, Linz, Austria) at 10 mg/ml and 1 μ mol/ml, respectively. 168 μ g/ml KLKL₅KLK was taken as 100 nmol/ml. CpG-ODN1668 with a phosphorothioate backbone (5'-TCCATGACGTTCCGATGCT-3', Purimex, Göttingen, Germany) was used for comparison studies. All compounds had undetectable amounts of endotoxin as measured by the limulus amoebocyte assay (BioWhittaker, Aspen, Germany).

TAMRA-succinimidylester (Molecular Probes, Eugene, OR, USA) was attached at the N-terminus of KLKL₅KLK after peptide synthesis by labeling the peptide still attached to the polystyrene resin. The labeled KLKL₅KLK was cleaved from the resin in the presence of 95% TFA, 3% TES, 2% H₂O and purified by HPLC. Cy5-coupling at the 5'-end of ODN1a was obtained from Purimex (Göttingen, Germany).

2.4. Vaccination of mice

Vaccines were formulated by mixing antigens with IC31 or its single components as indicated in respective figure

legends using 10 mM Tris–HCl/270 mM sorbitol buffer (pH 7.5–8). Injection volume and injection sites are also given in the respective figure legends.

2.5. Cell preparation and purification

Vaccinated mice were sacrificed by cervical dislocation, spleens and lymph nodes were excised and single cell suspensions were prepared. Erythrocytes from the spleen preparation were removed by lysis in Tris–ammonium chloride buffer. Viable cell numbers were determined by trypan blue exclusion. Single cell suspensions from draining lymph nodes were prepared following this same protocol whereby the erythrocyte lysis was omitted.

Peripheral blood mononuclear cells (PBMC) were prepared from anesthetized mice by obtaining blood via the orbital vein. PBMC from heparinized blood were isolated by Ficoll gradient centrifugation (density 1.077 g/ml, PAA Laboratories, Linz, Austria).

CD4⁺ Th cells from DO11.10 mice were isolated from spleen single cell suspensions by positive selection using magnetic activated cell sorting (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated cells was evaluated by flow cytometry.

All cells were cultured in complete medium, which is defined as DMEM (PAA Laboratories, Linz, Austria) supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 1 mM nonessential amino acids, 1× sodium pyruvate, 100 µg/ml gentamicin (all from PAA Laboratories, Linz, Austria) and 50 µM 2-mercaptoethanol (Life Technologies, Paisley, UK).

2.6. ELISPOT assays

IFN-γ ELISPOT assays were performed as described previously [16]. For IL-4 ELISPOT assays, 11B11 (2 µg/ml, BD Biosciences, San Jose, CA, USA) was used as coating antibody and biotinylated BVD6-24G2 (1 µg/ml, BD Biosciences, San Jose, CA, USA) as detection antibody. The same procedure as for IFN-γ ELISPOT assay was followed except that cells were stimulated for 48 h. Concanavalin A (ConA, 10 µg/ml, Amersham Biosciences AB, Uppsala, Sweden) was used as positive control for IFN-γ ELISPOT and PMA/Ionomycin as positive control for IL-4 (2×10^{-8} M/ 7.5×10^{-7} M, both Sigma–Aldrich, Austria).

ELISPOT plates were analyzed using BIOREADER 2000 (BioSys, Karben, Germany). Results are expressed as the number of cytokine producing cells per 1×10^6 unseparated spleen cells or PBMC $\pm$ S.D.

2.7. CFSE labeling of murine spleen cells

Purified spleen cells were washed in PBS and resuspended in PBS at 2×10^7 cells/ml. CFSE (5- (and 6-) carboxyflu-

orescein diacetate succinimidyl ester (stock solution: 5 mM in DMSO, Molecular probes, Eugene, OR, USA) was diluted in PBS and mixed with the cell suspension. After incubation for 5 min at 37 °C/5% CO₂, the labeling reaction was stopped by the addition of pure FCS to a final concentration of 5% (v/v). Cells were washed, counted and used either for the in vivo CTL assay or for the in vitro T cell proliferation assay.

2.8. In vivo CTL assay

To prepare target cells for the evaluation of cytotoxicity of vaccination-induced T cells, single spleen cell suspensions from syngeneic naïve mice were prepared as described above. Spleen cells were divided into the following populations: (1) cells pulsed with the peptide that was used for immunization (in case of injection with OVA, OVA_{257–264} was used as relevant peptide antigen) and labeled with a high concentration of CFSE (2.5 µM); (2) cells pulsed with an irrelevant peptide and labeled with an intermediate concentration of CFSE (0.25 µM); (3) unpulsed spleen cells labeled with a low concentration of CFSE (0.025 µM). Cells were pulsed with 10 µg/ml of the respective peptides for 60 min at 37 °C/5% CO₂. Finally, 1×10^7 cells of each population were mixed in 100 µl PBS per mouse and adoptively transferred into vaccinated mice by i.v. injection. In vivo cytotoxicity was determined for individual mice by collecting draining (popliteal) lymph nodes approximately 20 h after transfer. Single cell suspensions from lymph nodes were prepared and the presence of the three different target cell populations was analyzed in individual mice by flow cytometry using a FACSCalibur® flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA, USA).

The percentage of peptide-specific lysis was calculated according to following formula: %lysis = $(1 - \%CFSE^{high} / \%CFSE^{low}) \times 100$; the percentage of unspecific lysis was calculated according to following formula: %lysis = $(1 - \%CFSE^{medium} / \%CFSE^{low}) \times 100$. The percentages of CFSE^{high}, CFSE^{medium}, CFSE^{low} were calculated by flow cytometer. Results are expressed as the mean percentage of cell lysis observed for each experimental group $\pm$ S.D.

2.9. Determination of antigen-specific antibody levels

Antigen-specific total IgG, IgG1 and IgG2b antibodies in the sera of OVA-vaccinated mice were determined by a sandwich ELISA. Since the IgG2a encoding gene is deleted in C57BL/6 mice [21], only IgG2b was detected in these mice. At day 21 after injection, mice were bled and sera of each experimental group were pooled. Maxisorp microtiter plates (Nunc Immuno-plate, Denmark) were coated overnight with 10 µg/ml OVA and serial dilutions of sera were applied. Bound OVA-specific antibodies were detected by sequential incubation with biotinylated anti-mouse IgG(H+L), IgG1 (Southern Biotechnologies Associates, Birmingham, AL,

USA) or IgG2b antibodies (BD Biosciences, San Jose, CA, USA) and streptavidin-peroxidase conjugate (Roche Diagnostics, Vienna, Austria) followed by ABTS substrate solution (Sigma–Aldrich, Austria). The color intensity was measured at 405 nm with a Tecan Sunrise microplate reader (Tecan, Salzburg, Austria). The titer (1:x) is calculated from the serum dilution leading to half-maximal OD.

2.10. Analysis of the *in vivo* distribution of fluorescently labeled vaccine compounds

A 100 µg fluorescein (FI)-labeled OVA_{257–264} was injected s.c. into the flank of C57BL/6 mice along with 16.8 µg (=10 nmol) TAMRA-labeled KLKL₅KLK and/or 5 nmol Cy5-labeled ODN1a. At the indicated time points, the inner side of the injection site was inspected by eye for the presence of the fluorescently labeled compounds. Digital images were taken and analyzed using Adobe Photoshop 7.0.

2.11. Preparation of BMDC

Bone marrow-derived dendritic cells (BMDC) were prepared as previously described [22] with slight modifications. Briefly, femurs and tibiae from naïve mice were flushed with DMEM. Bone marrow cell suspensions were passed through a 70-µm nylon filter, centrifuged, and resuspended in a Tris–ammonium chloride buffer to lyse erythrocytes. CD4⁺ (GK1.5, ATCC, TIB-207), CD8⁺ (2.43, ATCC, TIB-210), Gr-1⁺ (RB6-8C5, kindly provided by Dr. R. Coffman, DNAX, USA) and MHC class II⁺ (M5/114.15.2, ATCC, TIB-120) cells were removed by antibody-mediated complement lysis. Bone marrow precursor cells were then purified by Ficoll gradient centrifugation (density 1.077 g/ml, PAA Laboratories, Linz, Austria), counted and plated in 24-well plates at 1.5×10^6 cells/well in complete DMEM medium supplemented with 10% (v/v) GM-CSF supernatant derived from the GM-CSF producing cell line X6310-GM-CSF (kindly provided by Dr. T. Decker, Vienna, Austria) in 24-well plates. At day 3 of culture, culture medium was completely exchanged. At day 6 of culture, BMDC were harvested and stimulated with IC31 and used *in vitro* for phenotypic and functional assays.

2.12. Phenotypic analyses of IC31-stimulated BMDC

BMDC were prepared as described above and were cultured for 18 h at 10^6 cells/1 ml in complete DMEM medium supplemented with GM-CSF in flat-bottom 24-well plates in the presence of IC31 (10 nmol/ml KLKL₅KLK and 0.5 nmol/ml ODN1a). After incubation, cells were harvested and stained with anti-CD11b-APC (M1/70), anti-CD11c-PE (HL3), anti-MHC class II-FITC (2G9), anti-CD40-FITC (3/23), anti-CD54-biotin (3E2), anti-CD80-biotin (16-10A1), anti-CD86-biotin (GL1), streptavidin-PerCP (all

BD Biosciences, San Jose, CA, USA) or anti-MHC class I-Alexa 488 (M1/42.3.9.8.HLK, ATCC, TIB-126). Analyses were performed by flow cytometry using a FACSCalibur[®] flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA, USA). Typically, cell aggregates and dead cells were segregated from living cells by plotting size (FSC) against granularity (SSC) and setting a gate to exclude small and not granulated cells. A total of 10,000 events of gated living cells were acquired.

2.13. *In vitro* proliferation and differentiation of T cells

In vitro generated BMDC were stimulated with IC31 as described for the phenotypic analysis and pulsed in parallel with 10 µg/ml OVA for 18 h at 1×10^6 cells/ml/well in flat-bottom 24-well plates in DMEM supplemented with 10% (v/v) GM-CSF supernatant. Following a washing step, BMDC (1×10^5) were cultured in 24-well plates with purified CFSE-labeled (at 2.5 µM) CD4⁺ TCR transgenic Th cells (1×10^6 cells/well) for 4 days and analyzed by flow cytometry to evaluate T cell proliferation. Magnetic sorting of CD4⁺ Th cells is described in *Cell preparation and purification*. Analyses were performed by flow cytometry using a FACSCalibur[®] flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA, USA).

To determine the amounts of IL-4 and IFN-γ produced by differentiated CD4⁺ Th cells, unlabeled CD4⁺ Th cells were co-cultured with IC31-stimulated BMDC pulsed with OVA (10 µg/ml). For re-stimulation on day 3, the cells of each individual sample with different stimulation were harvested, counted and re-plated at 2×10^5 cells/well in 96-well plates coated with anti-CD3 (145-2C11, 5 µg/ml) and anti-CD28 (37.51, 10 µg/ml) monoclonal antibodies (kindly provided by Dr. E. Schmitt, Mainz, Germany) for re-stimulation. After 24 h of incubation at 37 °C, supernatants were collected and stored at –20 °C until they were analyzed by ELISA.

2.14. Cytokine ELISAs

To determine the levels of IFN-γ and IL-4 in cell culture supernatants, microtiter plates (Maxisorp, Nunc Immunoplate, Denmark) were coated with purified capture mAb, namely anti-IFN-γ (R4-6A2) and anti-IL-4 (BVDA4-11D11) (both from BD Biosciences, San Jose, CA, USA). Biotinylated anti-IFN-γ, (AN18.17.24, kindly provided by Dr. E. Schmitt, Mainz, Germany) and anti-IL-4 (BVD6-S4G2, BD Biosciences, San Jose, CA, USA) were used for the detection of IFN-γ and IL-4 binding, respectively. The read-out was then obtained by incubating plates with streptavidin-HRP conjugate (Roche Diagnostics, Vienna, Austria), followed by ABTS substrate solution (Sigma–Aldrich, Austria). The color intensity was measured at 405 nm with a Tecan Sunrise microplate reader (Tecan, Salzburg, Austria).

3. Results

3.1. IC31 induces peptide-specific cellular type 1 immune responses in vivo

It was recently shown that the co-injection of the cationic peptide poly-L-arginine and the negatively-charged CpG-ODN in combination with an antigen resulted in increased and prolonged antigen-specific immune responses when compared to injection of antigen alone or antigen in combination with either poly-L-arginine or CpG-ODN [16]. We hypothesized that other cationic peptides in combination with ODNs might show a similar immunostimulatory effect. Meanwhile, a new cationic antimicrobial peptide KLKL₅KLK was described in our laboratory and shown to possess type 2-inducing capacities [32]. Besides, we developed a novel synthetic immunostimulatory single stranded oligodeoxynucleotide (ODN1a) containing deoxy-Inosine/deoxy-Cytosine repeats linked by a phosphodiester backbone. In this study, the combination of the two compounds, designated IC31, was analyzed for its immunostimulatory capacity.

In order to evaluate the potency of IC31 to induce peptide-specific immune responses in vivo, either the ovalbumin-derived peptide OVA_{257–264} or the murine tyrosinase-related protein 2-derived peptide TRP-2_{181–188} was co-injected with IC31 into C57BL/6 mice. One week after vaccination, immune responses were evaluated by enumerating peptide-specific IFN- γ -producing cells using an ELISPOT assay. As shown in Fig. 1A, co-injection of IC31 with OVA_{257–264} induced a high number of peptide-specific IFN- γ -producing cells, whereas injection of OVA_{257–264} alone or with either KLKL₅KLK or ODN1a, the single compounds of IC31, did not induce peptide-specific IFN- γ -producing cells. Similar results were obtained using the peptide TRP-2_{181–188} as antigen (Fig. 1B). The induction of peptide-specific IL-4 production could not be observed under these experimental conditions (data not shown).

Thus, these data indicate that IC31 represents a novel potent immunostimulator inducing peptide-specific cellular type 1 responses, characterized by IFN- γ production.

3.2. IC31 induces a type 1 cellular and a mixed type 1/2 humoral response to OVA protein in vivo

Since peptide-induced responses are independent from antigen uptake and processing, the effect of IC31 on an immune response against a whole protein antigen was also investigated using ovalbumin (OVA) as a model protein by measuring OVA-specific cellular and humoral immune responses. For this purpose, C57BL/6 mice were injected once with OVA or OVA/IC31 and data were collected at day 21 after injection. Cellular type 1 and 2 responses were evaluated by performing IFN- γ and IL-4 ELISPOT assays, respectively. Antigen-specific cytokine-producing cells in a pool of splenocytes of each experimental group were deter-

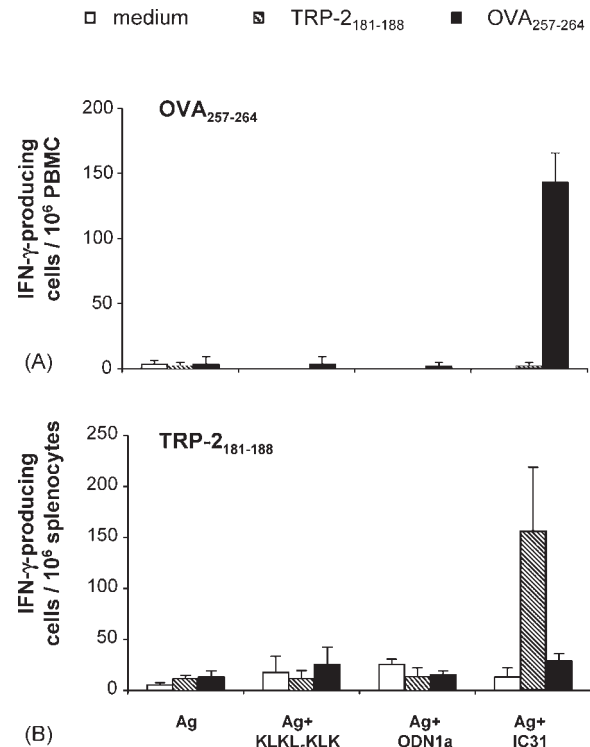


Fig. 1. IC31-based vaccines prime peptide-specific T cells in vivo. C57BL/6 mice (5/group) were injected into hind footpads (50 μ l/footpad) either with OVA_{257–264} peptide (A) or TRP-2_{181–188} (B) alone (100 μ g peptide/mouse) or in combination with KLKL₅KLK (100 nmol/mouse), ODN1a (5 nmol/mouse) or IC31 (KLKL₅KLK and ODN1a). 10 mM Tris/270 mM sorbitol buffer was used for the formulation of the vaccine. At day 7 after injection, PBMC (A) or splenocytes (B) were prepared and analyzed for peptide-specific IFN- γ -producing cells using ELISPOT assay as described in Section 2. For restimulation were used specific peptides (used for vaccination) TRP-2_{181–188} (A), OVA_{257–264} (B) and unspecific peptides OVA_{257–264} (A), TRP-2_{181–188} (B), all at 10 μ g/ml. Medium was used as background control. For each experimental group, PBMC (A) (1×10^5 /well) or splenocytes (B) (3×10^5 /well) were plated in triplicates in 96-well ELISPOT plates. The number of peptide-specific IFN- γ -producing cells in a pool of 1×10^6 cells as the mean of triplicates $\pm$ S.D. is shown. Similar results were obtained in at least four independent experiments.

mined by re-stimulation with (a) the whole protein OVA as well as with (b) the known H-2K^b restricted MHC class I epitope OVA_{257–264} and (c) the I-A^b restricted MHC class II epitope OVA_{265–280}.

As shown in Fig. 2A, co-injection of IC31 induced IFN- γ -producing cells detectable upon re-stimulation with either OVA or both OVA-derived peptides. The highest number of antigen-specific cells was induced against the MHC class I epitope OVA_{257–264}. The number of OVA-induced IL-4-producing cells was only slightly increased upon co-injection of OVA with IC31, independent of the type of antigen used for re-stimulation. Further analyses revealed that neither IFN- γ - nor IL-4-producing cells were induced upon co-injection of OVA with either ODN1a or KLKL₅KLK alone (data not shown), clearly showing that the potent immunostimulatory capacity of IC31 is based on the synergistic effect of its two components. Moreover, the resulting immune response

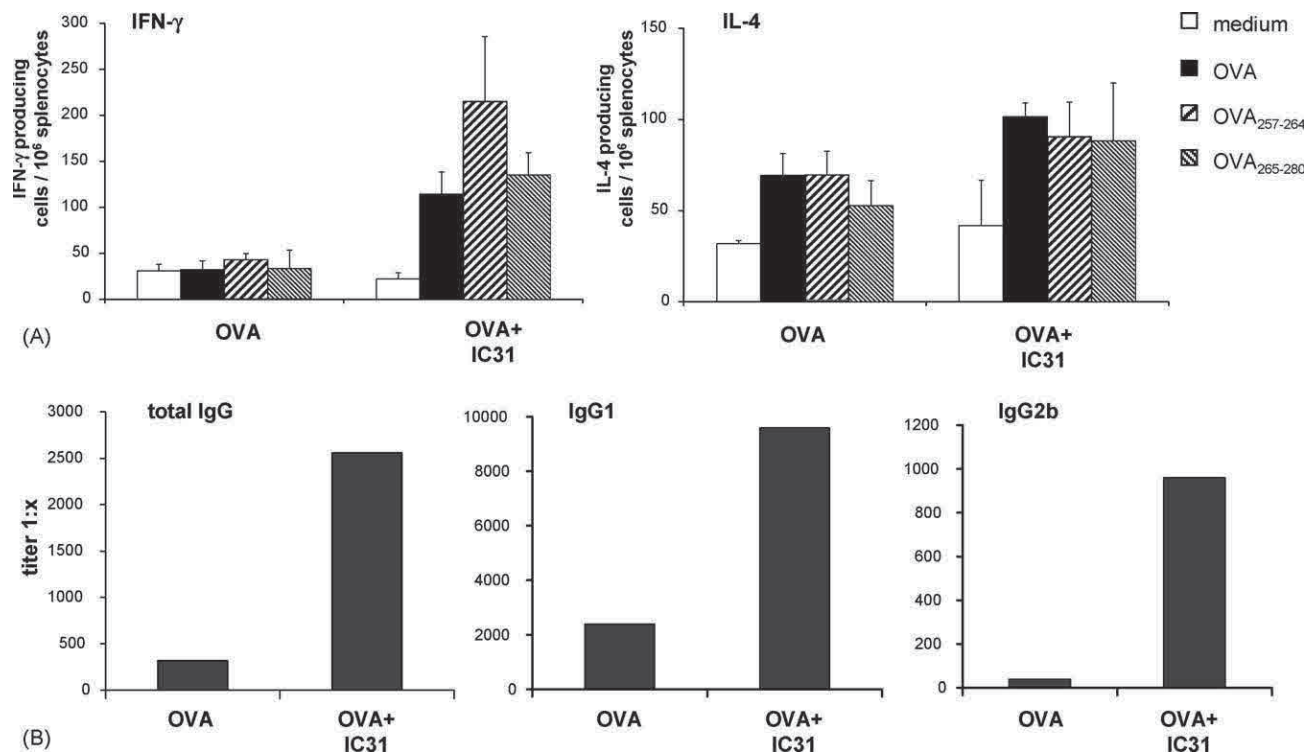


Fig. 2. Mice vaccinated with IC31/OVA respond by protein-specific cellular and humoral types 1/2 responses. C57BL/6 (5/group) were injected into hind footpads (50 μ l/footpad) at day 0 with OVA (100 μ g/mouse) alone or in combination with IC31 (100 nmol KLKL₅KLK and 4 nmol ODN1a/mouse). A 10 mM Tris/270 mM sorbitol buffer was used for the formulation of the vaccine. OVA-specific cellular and humoral immune response was analyzed at day 21 after vaccination. (A) Spleen cells were prepared and analyzed for the presence of IFN- γ - and IL-4-producing cells using respective ELISPOT assays (described in Section 2). For restimulation were used OVA (50 μ g/ml), OVA₂₅₇₋₂₆₄ and OVA₂₆₅₋₂₈₀ peptides (10 μ g/ml) or medium alone as a background control. For each experimental group, 3×10^5 (IFN- γ -ELISPOT) or 1×10^6 splenocytes/well (IL-4-ELISPOT) were plated in triplicates in 96-well ELISPOT plates. The number of antigen-specific IFN- γ - or IL-4-producing cells in a pool of 1×10^6 splenocytes as mean of triplicates $\pm$ S.D. is shown. (B) To determine OVA-specific antibody titers, sera in each experimental group were pooled and analyzed by ELISA as described in Section 2. Levels of OVA-specific total IgG, IgG1 and IgG2b antibodies were determined. Similar results were obtained in at least four independent experiments.

directed against MHC class I as well as MHC class II epitopes upon co-injection of the whole protein OVA and IC31 also indicates that IC31 exerts stimulatory effects on antigen uptake, processing and presentation by APC in vivo.

The IC31-induced enhancement of the immune response to OVA protein was also confirmed by the analysis of the humoral response. For this purpose, sera from C57BL/6 mice were analyzed by ELISA for the presence of OVA-specific total IgG as well as different IgG subtypes (IgG1 as indicator of a type 2 response and IgG2b as indicator of a type 1 response). As shown in Fig. 2B, OVA-specific total IgG as well as both IgG1 and IgG2b, were strongly increased upon co-injection with IC31 compared to injection of OVA alone.

Taken together, these data show that IC31 induces very potently mainly type 1 cellular responses to OVA protein (both, MHC class I- and MHC class II-restricted), whereas the induced humoral immune response is characterized by a mixed type 1/2 phenotype.

3.3. IC31 induces potent antigen-specific CTL in vivo

One objective for the development of IC31 was the induction of potent cytotoxic effector T lymphocytes (CTL) that are

able to eliminate target cells in an antigen-specific manner. Induction of CTL was measured using an in vivo CTL assay adapted from Oehen and Brduscha-Riem [23]. The adoptive transfer of fluorescently-labeled target cells pulsed with relevant antigen into vaccinated mice allows the determination of the lytic potential of vaccine-induced antigen-specific CTL directly in vivo. As controls were used fluorescently-labeled target cells – unpulsed or pulsed with an irrelevant antigen.

Target cells were adoptively transferred on day 7 after vaccination. CTL response was determined by flow cytometry one day after injection of target cells. The results obtained reveal that upon co-injection of TRP-2₁₈₁₋₁₈₈ and IC31 a strong TRP-2₁₈₁₋₁₈₈-specific CTL response was induced leading to the specific lysis of target cells in vivo (Fig. 3 and Table 1). No cytotoxic activity was observed in naïve mice or mice vaccinated with peptide alone. Similar effects were detected using OVA₂₅₇₋₂₆₄ or the whole OVA protein for vaccination (Table 1).

Thus, besides the induction of antigen-specific cytokine-producing cells, IC31 is also able to induce potent CTL effector cells that are capable to kill target cells in vivo in a highly specific manner.

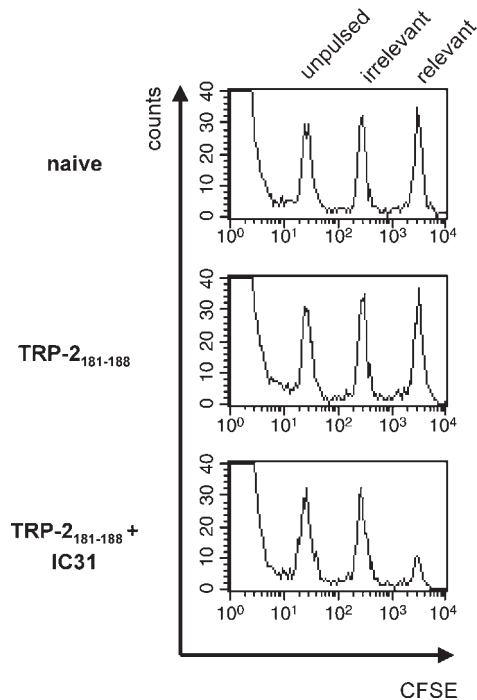


Fig. 3. Antigen-specific CTL are induced by IC31 in vivo. C57BL/6 (5/group) were injected into hind footpads (50 μ l/footpad) with TRP-2_{181–188} peptide (100 μ g/mouse) alone or in combination with IC31 (100 nmol KLKL₅KLK and 5 nmol ODN1a/mouse). Naïve mice were used for control purposes. A 10 mM Tris/270 mM sorbitol buffer was used for formulation of the vaccine. At day 7 after vaccination, CFSE-labeled target cells either unpulsed or pulsed with a relevant (TRP-2_{181–188}, 10 μ g/ml) or irrelevant peptide (OVA_{257–264}, 10 μ g/ml) were adoptively transferred (1×10^7 cells of each population) by i.v. injection into vaccinated or naïve control mice as described in Section 2. On day 8 after vaccination and one day after transfer of target cells, popliteal lymph node cells of single mice were prepared and analyzed by flow cytometry. Shown are histograms of individual mice, which are representative of each experimental group. Three significant peaks representing the respective target cell population are detectable. The left peak represents the unpulsed target cell population labeled with 0.025 μ M CFSE; the central peak represents target cells pulsed with irrelevant OVA_{257–264} peptide and labeled with 0.25 μ M CFSE and finally, the right peak represents the target cell population pulsed with the relevant TRP-2_{181–188} peptide and labeled with 2.5 μ M CFSE. Similar results were obtained in at least two independent experiments.

Table 1
Antigen-specific CTL are induced by IC31 in vivo

	Naïve ^a (%)	Ag (%)	Ag + IC31 (%)
TRP-2 _{181–188} ^b	21.3 $\pm$ 3.0 ^c	18.9 $\pm$ 5.0	69.2 $\pm$ 11.5
OVA _{257–264}	8.4 $\pm$ 5.2	7.3 $\pm$ 6.0	60.4 $\pm$ 5.4
OVA	4.2 $\pm$ 4.4	9.1 $\pm$ 8.0	73.5 $\pm$ 24.0

^a Five C57BL/6 mice per experimental group were injected with antigen alone or in combination with IC31 (100 nmol KLKL₅KLK/5 nmol ODN1a per mouse). Naïve mice were used for control purposes. At day 7 after injection, an in vivo CTL assay was performed as described in Fig. 3 and Section 2.

^b Peptide or protein antigens used for injection at 100 μ g per mouse.

^c Antigen-specific lysis efficiency (%) was determined using draining lymph node cells and was calculated as described in Section 2. Unspecific lysis of target cells pulsed with irrelevant antigen was not higher than 15% (data not shown). Similar results were obtained in at least six (OVA_{257–264}, OVA) and two (TRP-2_{181–188}) independent experiments.

3.4. IC31 signaling involves the TLR9/MyD88-dependent pathway

It has been described that DNA molecules containing CpG-motifs exert their immunostimulatory effect via the TLR9/MyD88 signaling pathway [8]. Since ODN1a, one component of IC31, is a synthetic single stranded oligodeoxynucleotide (ODN), we were interested to analyze the dependency of the immunostimulatory effects of IC31 on the presence of TLR9 and MyD88. For this purpose, the immune response induced upon co-injection of OVA and IC31 in wild type C57BL/6 mice was compared to that induced in TLR9 knock-out as well as MyD88 knock-out mice. Three weeks after a single injection of OVA and IC31, the immune response against the MHC class I-restricted epitope OVA_{257–264} and the MHC class II-restricted epitope OVA_{265–280} was evaluated using an IFN- γ ELISPOT assay.

Compared to injection of OVA alone, co-injection of OVA and IC31 into wild type C57BL/6 mice strongly increased immune response against OVA_{257–264} and OVA_{265–280} (Fig. 4). Interestingly, this antigen-specific immune response induced by IC31 was completely abrogated in both TLR9 and MyD88 knock-out mice. Similar results were obtained when

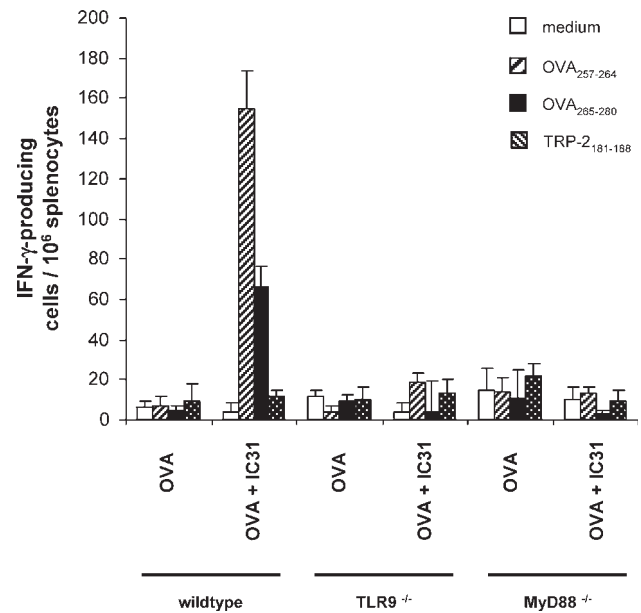


Fig. 4. IC31 signals via TLR9- and MyD88-dependent pathway. C57BL/6, TLR9 knock-out or MyD88 knock-out mice (5/group) were injected into hind footpads (50 μ l/footpad) with OVA (100 μ g/mouse) alone or in combination with IC31 (100 nmol KLKL₅KLK and 5 nmol ODN1a/mouse). A 10 mM Tris/270 mM sorbitol buffer was used for formulation of the vaccines. At day 7 after injection, splenocytes were prepared and analyzed for the presence of IFN- γ -producing cells using ELISPOT assay (Section 2). For restimulation, specific (OVA_{257–264} or OVA_{265–280}) or unspecific peptides (TRP-2_{181–188}) were used at 10 μ g/ml. For each experimental group, 3×10^5 splenocytes/well were plated in 96-well ELISPOT plates in triplicates. The number of antigen-specific IFN- γ -producing cells in a pool of 1×10^6 splenocytes as the mean of triplicates $\pm$ S.D. is shown. Similar results were obtained in three independent experiments.

OVA was used in combination with CpG-ODN 1668 ([8] and data not shown).

Thus, these data clearly indicate that IC31, composed of the deoxy-Inosine/deoxy-Cytosine containing ODN1a and KLKL₅KLK, stimulates an immune response by engaging the TLR9/MyD88-dependent signaling pathway.

3.5. Co-administration of antigen and IC31 induces depot formation

The interaction of the two components of IC31, KLKL₅KLK and ODN1a, may be responsible for the formation of a depot in a similar manner as recently described for the combination of poly-L-arginine and CpG-ODN [16]. In order to investigate this assumption, a vaccine with fluorescently-labeled compounds – containing OVA_{257–264}-FI, KLKL₅KLK-TAMRA, ODN1a-Cy5 – was formulated and injected s.c. into the flank of mice. At days 1, 7, 28 and 58 after administration, injection sites were inspected for the presence of labeled compounds.

As shown in Fig. 5, when OVA_{257–264}-FI was co-injected with KLKL₅KLK-TAMRA and ODN1a-Cy5, all three components formed a depot at the injection site, which was still detectable at day 58 after injection (last time point analyzed). Upon injection of OVA_{257–264}-FI in combination with ODN1a-Cy5 the components rapidly diffused from the injection site. In contrast, co-injection of OVA_{257–264}-FI with KLKL₅KLK-TAMRA indicates that the presence of KLKL₅KLK was needed for depot formation. However, the presence of all vaccine components seems to stabilize the depot formation as demonstrated by a more prominent depot at later time points.

Table 2
Activation of BMDC by IC31

	Medium ^a (%)	IC31 ^a (%)
CD40 ^b	8.8 ^c	19.2
CD54	17.8	63.0
CD80	31.0	44.4
CD86	34.5	49.1
MHC I	68.0	76.5
MHC II	98.0	98.9

^a Stimulation of BMDC (1×10^6 cells/well in a 24 well plate) with medium or IC31 (10 nmol/ml KLKL₅KLK and 0.5 nmol/ml ODN1a) for 18 h at 37 °C/5% CO₂.

^b Surface molecule expression was analyzed by incubating BMDC with anti-MHC I-Alexa, anti-MHC II-FITC, anti-CD11c-PE and anti-CD11b-APC, anti-CD40-FITC, anti-CD54-biotin, anti-CD80-biotin and anti-CD86-biotin. SA-PerCP was used in a second-step reagent. Adequate isotypes were used as controls.

^c Analyses of living cells only (=100%) gated in the FSC:SSC dot plot.

3.6. IC31-activated BMDC stimulate proliferation and differentiation of TCR transgenic CD4⁺ Th cells in vitro

Among professional APC, dendritic cells (DC) are essential linkers of the innate and adaptive immune system in that they are specialized in capturing and presenting antigen as well as activating antigen-specific naïve T lymphocytes [24–26]. The potency of DC in this crucial step is dependent on expression of co-stimulatory molecules and/or secretion of certain cytokines required to optimally stimulate T cells as well as to influence the character of the resulting immune response.

Therefore, we investigated whether IC31 activates murine bone marrow-derived DC (BMDC). As shown in Table 2, IC31 up-regulated the surface expression of co-stimulatory

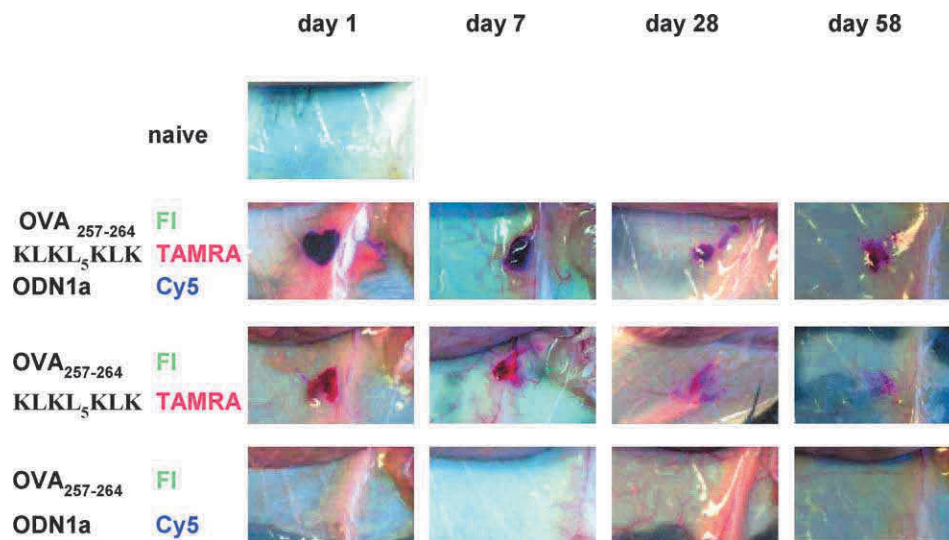


Fig. 5. IC31 mediates the formation of a depot at the injection site. C57BL/6 (3/group) were injected s.c. (100 μ l/mouse) into the right flank either with OVA_{257–264}-FI/KLKL₅KLK-TAMRA/ODN1a-Cy5, OVA_{257–264}-FI/KLKL₅KLK-TAMRA or OVA_{257–264}-FI/ODN1a-Cy5. The vaccine components were used at the following concentrations: 100 μ g OVA_{257–264}-FI, 16.8 μ g/ml KLKL₅KLK-TAMRA and 5 nmol ODN1a-Cy5 per mouse. 10 mM Tris/270 mM sorbitol buffer was used for formulation of the vaccine. At indicated time points, injection sites from different mice were inspected for the presence of labeled compounds at the inner side of the skin. Naïve mice were used as a control. Images were taken with a digital camera. Figure shows single mice as representative of each experimental group. Similar results were obtained in two independent experiments.

molecules (CD40, CD54, CD80 and CD86) and MHC class I molecules on BMDC. The most pronounced upregulation was seen for CD40 and CD54. MHC class II expression was already so high on BMDC in the medium control (98%) that further increase in the presence of IC31 could not be detected.

Furthermore, we investigated whether such IC31-activated BMDC were able to promote antigen-specific T cell proliferation in vitro using TCR transgenic CD4⁺ Th cells derived from DO11.10 mice as well established system. For this purpose, BMDC were pulsed overnight with OVA antigen in the presence or absence of IC31 to cause the presentation of MHC class I and class II OVA-derived epitopes on their cell surface. Following this step, IC31-activated/OVA-pulsed BMDC were co-cultured with fluorescently-labeled OVA_{323–339}-specific TCR transgenic CD4⁺ Th cells. At day 4 of incubation, T cell proliferation was determined by flow cytometry in a dye dilution assay as described in Section 2. As shown in Fig. 6, only IC31-activated/OVA-pulsed BMDC induced antigen-specific T cell proliferation with four cell divisions. In contrast, IC31-stimulated BMDC, OVA-pulsed un-stimulated BMDC and un-stimulated, un-pulsed BMDC had no effect on T cell proliferation.

In summary, these data indicated that IC31 promoted the induction of an immune response by activating BMDC, which in turn enabled the induction of T cell proliferation. In addition, BMDC incubated with OVA alone had no stimulatory effects. Since such BMDC can stimulate antigen-specific T

cell proliferation in the presence of OVA, it is most likely that antigen uptake and processing is promoted in the presence of IC31.

Next, the in vitro differentiation of OVA_{323–339}-specific TCR transgenic CD4⁺ Th cells (DO11.10) under the influence of IC31-treated BMDC was analyzed. For antigen-specific activation, T cells were co-cultured with OVA-pulsed and IC31-stimulated BMDC. After 48 h of incubation, cells were re-stimulated for additional 24 h in an antigen-independent way using plate-bound anti-CD3 and anti-CD28 antibodies. Release of IFN- γ and IL-4 into the supernatant was analyzed by ELISA. Under the chosen experimental conditions, T cells co-cultured with un-stimulated BMDC (medium) already produced low levels of IFN- γ and IL-4, that were slightly enhanced when BMDC were pre-incubated with OVA (Fig. 7). However, in the presence of IC31, OVA-pulsed BMDC reached a significantly higher level of activation to promote the development of Th1 and Th2 cells as evidenced by the antigen-specific production of IFN- γ and IL-4.

Taken together, IC31 not only stimulates BMDC, but also makes them capable to induce proliferation as well as differentiation of antigen-specific T cells. The data generated in vitro using the OVA_{323–339}-specific system show the induction of a mixed type 1/2 cellular response which also reflects the in vivo observation when OVA was co-injected with IC31.

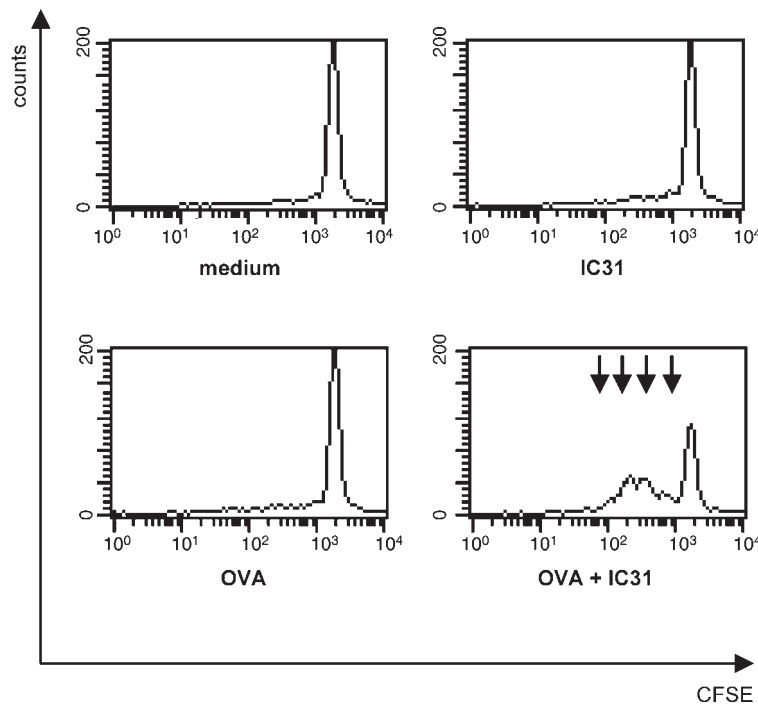


Fig. 6. IC31-activated BMDC induces antigen-specific proliferation of CD4⁺ TCR transgenic Th cells. BMDC (1×10^6 cells/well) were incubated in 24-well plates for 18 h at 37 °C/5% CO₂ with either medium, IC31 (12.7 nmol/ml KLKL₅KLK and 0.5 nmol/ml ODN1a), OVA (10 μ g/ml) or IC31/OVA. CD4⁺ Th cells were isolated from splenocytes of DO11.10 TCR transgenic mice by magnetic sorting. After a washing step, 1×10^5 BMDC/well were cocultured with 1×10^6 CFSE (2.5 μ M)-labeled CD4⁺ TCR transgenic Th cells/well in 24-well plates. At day 4 of culture, cells were collected and immediately analyzed by flow cytometry. CFSE fluorescence intensities of living lymphocytes, gated based on FSC:SSC profiles, were analyzed. Details of experimental settings are described in Section 2. Similar results were obtained in four independent experiments.

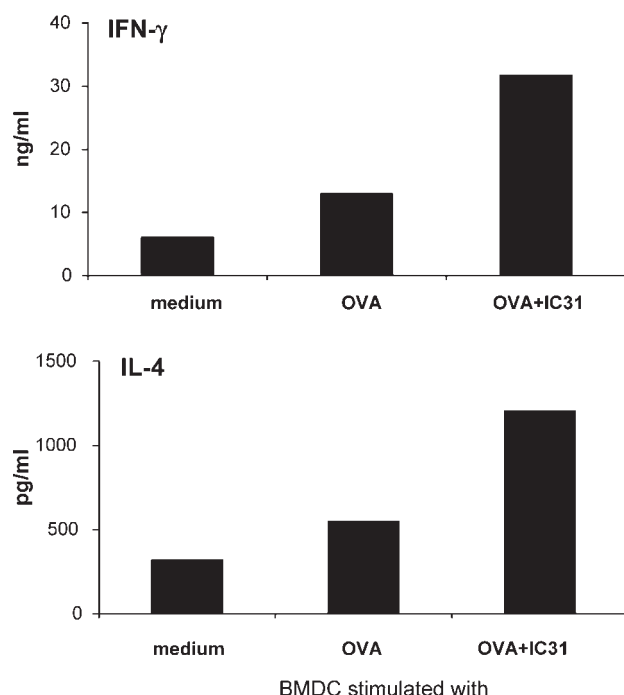


Fig. 7. Th cells differentiate into both Th1 and Th2 cells upon stimulation with IC31-activated BMDC. BMDC (1×10^6 cells/well in a 24 well-plate) were incubated for 18 h at $37^\circ\text{C}/5\% \text{CO}_2$ with medium, OVA protein (10 $\mu\text{g}/\text{ml}$) or IC31/OVA (12.7 nmol/ml KLKL₅KLK and 0.5 nmol/ml ODN1a). CD4⁺ Th cells were isolated from splenocytes of DO11.10 TCR transgenic mice by magnetic sorting. A 1×10^5 BMDC/well were co-cultured with 1×10^6 /well CFSE-labeled CD4⁺ TCR transgenic Th cells in 24-well plates. At day 3, cells were collected, washed and restimulated with plate-bound anti-CD3/anti-CD28 mAbs (5 and 10 $\mu\text{g}/\text{ml}$) for 24 h at $37^\circ\text{C}/5\% \text{CO}_2$. Supernatants were analyzed by ELISA for the presence of IFN- γ and IL-4. Details of experimental settings are described in Section 2. Similar results were obtained in three independent experiments.

4. Discussion

IC31 is a novel fully synthetic bi-component adjuvant consisting of the antimicrobial peptide KLKL₅KLK and the phosphodiester-backboned single stranded ODN1a. The ODN does not contain CpG motifs, rather it is characterized by deoxy-Inosine/deoxy-Cytosine repeats. IC31 strongly induces antigen-specific cellular and humoral immune responses. By contrast, adjuvants currently available for application in man, like Alum and MF59, predominantly induce type 2 responses and do not induce CTL [6]. In this study, we demonstrate that potent antigen-specific CTL responses, essential for protective immunity against intracellular pathogens and cancer, are generated under the influence of IC31. Apart from cellular responses, strong protein-specific humoral responses are also induced by IC31, implying its potency as adjuvant in vaccines directed against extracellular pathogens. Therefore, IC31 may represent a powerful alternative to currently available adjuvants in both prophylactic and/or therapeutic vaccine applications.

Importantly, IC31 does not have or induce systemic toxic effects as determined in extensive toxicological studies in rats

(data not shown) and is locally very well tolerated. In addition, systemic production of pro-inflammatory cytokines, such as IL-6 and TNF- α , was not detectable upon IC31 administration (data not shown). This is in contrast to certain oligodeoxynucleotide sequences containing CpG motifs, which are described to induce systemic side effects [10,27].

KLKL₅KLK, one component of IC31, was recently described to induce type 2 immunity against the model protein OVA following a prime-boost vaccination approach [32]. ODN1a, the second component of IC31, does not activate the immune system by itself, most probably due to its short half-life based on its phosphodiester backbone. However, the interaction of these compounds leads – under the chosen experimental conditions – to potent immune responses. Peptide-specific immune enhancement is characterized by the induction of type 1 cellular immune responses detected by IFN- γ production. Responses induced by IC31 against whole protein OVA is dominated by a type I cellular response whereas the humoral response shows characteristics of a mixed type 1/2 immunity. The detailed mechanisms underlying these observations will need further investigations; however, our data indicate that ODN1a strongly contributes to the induction of type 1 immune responses.

Using knock-out mice, we provide evidence that IC31 exerts its immunostimulatory effects via the TLR9/MyD88-signaling pathway. Most probably, ODN1a, the deoxy-Inosine/deoxy-Cytosine containing component of IC31, is responsible for this effect. So far, the only described ligands for the intracellularly expressed TLR9 are CpG-containing ODNs [8,28]. However, the involvement of other TLR, especially TLR3 as a receptor for Inosine-containing RNA molecule poly(I:C) [9] or TLR7 as a receptor for ssRNA [30–32], has to be investigated, although they were described to respond to RNA, but not DNA molecules.

Besides providing a stabilizing effect for ODN1a through direct molecular interaction, the positively charged peptide KLKL₅KLK seems to have a delivery function for antigen and ODN1a. This assumption is in line with publications in the field of gene therapy, where polycationic substances including short synthetic peptides efficiently deliver DNA in vivo [27]. Furthermore, our previous findings of an improved immunostimulatory effect of CpG-ODN when injected with poly-L-arginine also support this assumption [16]. Whether IC31, as KLKL₅KLK/ODN1a complex, enters cells via the endocytic pathway, via calreticulin [29] or whether an unknown receptor or mechanism is involved remains to be investigated.

Another property of KLKL₅KLK contributing to the adjuvant effect of IC31 is the induction of a depot at the injection site. Following a single injection, this depot is detectable at least until day 58 and retains not only KLKL₅KLK and ODN1a, but also the antigen. Such a depot-inducing adjuvant effect is of great advantage since it might favour continuous, long-term antigen and adjuvant release to stimulate the permanent induction of antigen-specific immune responses [30]. Most likely, both depot formation and sustained activa-

Table 3
Characteristics of adjuvants

Adjuvant	Depot formation	Systemic IL-6, TNF- α	T cell response	CTL	B cell response
Alum	Yes	No	Type 2	No	IgG1
MF59	No	No	Type 2	No/yes	IgG1
CpG-ODN	No	Yes	Type 1	Yes	IgG2
IC31	Yes	No	Type 1/2	Yes	IgG1, IgG2

tion of APC followed by antigen uptake and processing are responsible for the induction of the observed potent IC31-mediated antigen-specific immune enhancement. It is also conceivable that IC31 may play a role in the partial protection of vaccine components against enzymatic degradation over a certain period of time.

The activation of DC, the most potent APC for stimulating naïve T cells is an important prerequisite for the induction of adaptive immune responses [24–26]. In this context, our in vitro studies have shown the direct effects of IC31 on DC. Activation of murine BMDC by IC31 renders them competent to strongly stimulate T cell proliferation and differentiation. Indeed, using OVA_{257–264}-specific TCR transgenic CD4⁺ Th cells, we could show that antigen-specific IFN- γ and IL-4 production was induced, perhaps reflecting the in vivo situation of a mixed type 1/2 immunity induced by IC31. Furthermore, the stimulatory effect of IC31 on antigen uptake, processing and presentation by APC in vivo is also indicated by the immune response directed against MHC class I as well as MHC class II epitopes upon co-injection of the whole protein OVA and IC31. This indicates that IC31 promotes the process known as cross-presentation that leads to the internalization of exogenous antigens by APCs, routed into the MHC class I presentation pathway followed by the priming of naïve CD8⁺ T cells [31].

Considering the signal theory [2,3,28,30] as an immunological concept of vaccine adjuvant activity, IC31 provides the following signals: (a) signal 0: activation of APC via TLR9/MyD88 signaling pathway representing PRR/PAMP (pathogen recognition receptor/pathogen-associated microbial patterns) interaction; (b) signal 1: upregulation of MHC molecules, thereby improving the presentation of antigens to T cells; (c) signal 2: upregulation of co-stimulatory molecules, providing the fully activated status of APC needed for appropriate stimulation of T cells. The depot formation induced by KLKL₅KLK further increases the co-stimulatory potency through the sustained presence of antigen and immunostimulatory adjuvant providing a constant antigen-specific stimulation of immune responses over a long period of time. Although signal 3 is described to lead to cytokine production by APC (e.g. IL-12) needed for stimulation and differentiation of T cells, we could not detect this in our system. Detailed analyses of cytokine production revealed that under the chosen experimental conditions no IL-12, TNF- α or IL-6 was detectable in the supernatant of these BMDC cultures with or without IC31 (data not shown). Nevertheless, it can be concluded that IC31 is a potent inducer of BMDC

activation and maturation in terms of the up-regulation of co-stimulatory and MHC class I molecules.

Table 3 summarizes the characteristic features of IC31 in comparison to other adjuvants such as Alum, MF59, and CpG-ODN [6] indicating its unique properties that fulfill the prerequisites of a successful vaccine adjuvant. IC31 seems to have all the advantages, but not the disadvantages of currently applied adjuvants that makes it a very attractive candidate adjuvant for different kinds of vaccine strategies where especially cell mediated immunity but also immune responses are required. Since IC31 is currently tested as adjuvant in a variety of subunit vaccines, reports about its potency to stimulate protective immunity in relevant animal models will be soon available.

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