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ORIGINAL ARTICLE

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Nanoparticles prepared from porcine cells support the healing of cutaneous inflammation in mice and wound re-epithelialization in human skin

Natalia Kunz[1] | Eva Hauenschild[1] | Sebastian Maass[1] | Kai-Uwe Kalies[2] | Matthias Klinger[1] | Melanie Barra[1] | Lars Hecht[3] | Franz Helbig[3] | Stefan Soellner[4] | Charles C. Caldwell[5] | Ralf J. Ludwig[6] | Jürgen Westermann[1] | Kathrin Kalies[1]

[1]Institute of Anatomy, University of Luebeck, Luebeck, Germany

[2]Institute of Biology, University of Luebeck, Luebeck, Germany

[3]Varicula Biotec GmbH, Teterow, Germany

[4]Clinic for Aesthetic-Plastic Surgery, Prien, Germany

[5]Division of Research, Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH, USA

[6]The Lübeck Institute of Experimental Dermatology, University of Luebeck, Luebeck, Germany

Correspondence
Kathrin Kalies, Institute of Anatomy, University of Luebeck, Luebeck, Germany.
Email: kalies@anat.uni-luebeck.de

Present address
Melanie Barra, PPD GmbH, Karlsruhe, Germany

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Abstract

Previous reports have demonstrated that cell-derived nanoparticles (CDNPs) composed of bovine or porcine protein complexes exerted therapeutic effects against viral infections and cancer in mice and humans. Based on these observations, we asked whether CDNPs would improve inflammatory skin disorders. To address this, we utilized two distinct mouse models of cutaneous inflammation: the autoimmune skin-blistering disease epidermolysis bullosa acquisita (EBA) as an example of an autoantibody-induced cutaneous inflammation, and *Leishmania major* (*L. major*) infection as an example of a pathogen-induced cutaneous inflammation. In both models, we observed that CDNPs increased mRNA expression of the Th2 cytokine IL-4. Clinically, CDNPs decreased inflammation due to EBA and increased *L. major*-specific IgG1 levels without major effects on infected skin lesions. In addition, CDNPs supported the growth of keratinocytes in human skin cultures. In vitro studies revealed that CDNPs were taken up predominantly by macrophages, leading to a shift towards the expression of anti-inflammatory cytokine genes. Altogether, our data demonstrate that treatment with porcine CDNPs may be a new therapeutic option for the control of autoimmune-mediated inflammatory skin disorders.

KEYWORDS

autoimmune skin-blistering disease, therapy, wound healing

1 | INTRODUCTION

The high prevalence of pathological cutaneous inflammation has made the identification of safe and effective anti-inflammatory dermal therapies a priority. Previous reports have indicated that injecting xenogeneic cell-derived nanoparticles (CDNPs, commercially available as Biocomplex-"reaction pattern in vertebrate cells"; BC-RiV) improved viral infections and cancer.[1] CDNPs are protein complexes composed of intracellular proteins. The most abundant proteins are annexin A1, annexin A5, actin, *14-3-3ε*, *14-3-3ζ*, galectin-3, heat-shock proteins 27 and 70, histones 2A and 2B and nucleolin.[2,3] In addition, CDNPs contain small amounts of RNAs, mostly of ribosomal origin.[1,4]

Nothing is known regarding the effects of porcine CDNPs on cutaneous inflammation in mice and humans. To address this, in vivo and in vitro assays have been established. First, a transient cutaneous inflammation in mice by injection of autoantibodies against mouse type

Natalia Kunz and Eva Hauenschild contributed equally to this work.

7 collagen (mCol7) was induced for treatment with CDNPs. In this model, epidermal blister formation representing the autoimmune skin-blistering disease epidermolysis bullosa acquisita (EBA) appears. Several factors that mediate the effector phase of the disease such as complement, cytokines/chemokines and neutrophils have been identified in previous studies.[5] Second, we asked whether CDNPs would interfere with a non-sterile cutaneous inflammation. This is of importance since promotion of an anti-inflammatory wound-healing response could disturb the clearance of pathogens and thereby favour an inflammatory development. A cutaneous infection of C57BL/6 mice with *L. major* induces a transient inflammation at the site of infection. The resulting lesion size is highly variable and depends on the cytokine milieu. A Th1 response activates macrophages and supports parasite killing. In contrast, a Th2 response activates B cells to produce anti-*L. major* IgGs and leads to the differentiation of anti-inflammatory M2 macrophages. Because both, IgG and M2 macrophages, are not able to control parasite growth, lesion size will increase.[6] Finally, and thirdly, to investigate the possibility that CDNPs could affect epithelial growth directly, human skin cultures were performed.[7]

In conclusion, treatment with porcine CDNPs induced an increase in mRNA expression of the Th2 cytokine IL-4 in draining lymph nodes in both models of inflammation: EBA and *L. major* infection. An improved wound healing in EBA but no obvious effects on cutaneous lesions induced by *L. major* were observed. In addition, CDNPs enhanced epithelial growth in human skin cultures suggesting a supportive role in the repair of skin barrier function. This data demonstrate that treatment with porcine CDNPs could be a safe therapeutic option to support healing of chronic endogenous cutaneous inflammation.

2 | METHODS

2.1 | Human skin biopsies and mice

Excess body skins from 4 women (24, 26, 28 and 54 years of age) undergoing elective cosmetic surgery were obtained after informed consent and ethics approval (# 06-109, approval by the Institutional Research Ethics Committee at the University of Luebeck). Mouse experiments were performed in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment (Kiel, Germany, # V312-72241.122-1 (105-10), (23/z/08) and (43-4/09)). 8- to 12-week-old female C57BL/6 mice were obtained from Charles River Breeding Laboratories and housed in the central animal facility of the University.

2.2 | CDNPs: preparation and characterization

CDNPs (BC-RiV) were produced under GMP endotoxin-free conditions by Varicula Biotec GmbH (Teterow, Germany) as previously described.[1,2] Briefly, CDNPs were isolated from embryonic porcine kidney cells (EFN-R, Friedrich-Loeffler-Institute, Insel Riems, Germany) after culture in nutrient-deprived media for 2 days. Cell sonicates were prepared, centrifuged and subjected to chloroform treatment to remove the membrane fractions. Finally, the remaining pellet containing the CDNPs was resolved in PBS. Protein concentration was determined by Pierce protein assay kit (Thermo Fisher Scientific) and adjusted to 150 μg/ml. To avoid interassay variability, each assay was performed with one CDNP preparation. Characterization of particularity and sizes were assessed by Nanoparticle Tracking Analysis (NTA) with a NanoSight LM10 (Malvern Instruments LTD., Worcestershire, UK). A protein concentration of 150 μg/ml yielded 10^{11} to 10^{12} particles with a size between 100 and 150 nm. To detect integrity of CDNPs, 1% Triton X-100 was added for removal of potential surrounding membranes. As controls, dog pancreatic rough microsomes (RM) that consist of membranes and ribosomes were prepared from endoplasmic reticulum. An RM suspension containing 2.8 equivalents per μl (e.g, for definition, see Ref. [8]) was diluted 1:10^4 in PBS[8] (Fig. S3).

2.3 | Scanning electron microscopy

Experiment details are reported in Appendix S1.

2.4 | Animal models and treatments

For induction of EBA, rabbit anti-mCol7 IgGs were prepared as previously described.[9] EBA was induced by a total of 4 s.c. injections of 5 mg rabbit anti-mCol7 IgGs every other day. The control group received normal rabbit IgG using the same schedule. Disease severity was determined at ten time points until 40 days after initial IgG injection and expressed as the percentage of body surface area affected by skin lesions. Ear skin lesions and the inguinal lymph node were collected on day 12 and at the end of the observation period at 40 days, respectively. For infection with *L. major* (MHOM/IL/81/FE/BNI), promastigotes were cultured and injected as previously described.[10] Disease development was evaluated weekly by measuring the swelling of the infected footpad. Blood, draining lymph nodes and infected footpad skin were collected 5 weeks after infection. To detect the *L. major*-specific IgG isotypes in the serum, IgG levels were assessed quantitatively using a sandwich ELISA according to the manufacturer's protocol (Bethyl Laboratories Inc., Montgomery, TX, USA). Mice received 50 μl CDNPs (7.5 μg protein; 5×10^9-10^{10} particles) every other day s.c. alternating into the right and left upper hind legs. Treatment with CDNPs started 16 days after the first IgG injection in the EBA model and 3 days before *L. major* infection.

2.5 | Human wound-healing organ culture assay

The "punch-within-a-punch" design was performed as previously described.[7] Skin punches were cultured in 1 ml serum-free WE medium with 100 μl of either CDNPs containing 15 μg proteins (10^{10}-10^{11} particles) or PBS. After 3 or 6 days in culture, skin punches were snap-frozen and stored at −80 °C until further analysis.

2.6 | Histological analysis

12 μm thick histological sections of mouse or human skin samples were stained with haematoxylin and eosin or

immunohistologically with mAB. Other details are reported in Appendix S1.

2.7 | Analysis of gene expression levels

Real-time RT-PCR was performed as previously described.[11,12] For further information, see Appendix S1.

2.8 | *In vitro* assays of peritoneal cells

Peritoneal cells were isolated from naive C57BL/6 mice for incubation with CDNPs. Other experiment details are reported in Appendix S1.

2.9 | Statistical analysis

The statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). Statistical significance was assessed by Mann-Whitney *U* test (for unpaired samples), Wilcoxon signed rank test (for paired cell culture samples) or ANOVA (either one-way or repeated measures) with Newman-Keuls post hoc test. A *P* value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | CDNPs enhance Th2 immunity and promote clearance of experimental EBA

To establish a model for cutaneous wound healing of inflamed skin in vivo, we induced an autoimmune inflammation response by injecting rabbit IgGs specific for mouse type 7 collagen (mCol7).[9] The process of lesion formation during the first 12 days after injection of anti-mCol7-specific IgGs is well established: (i) the transferred anti-mCol7-specific rabbit IgGs bind to the DEJ, (ii) complement is

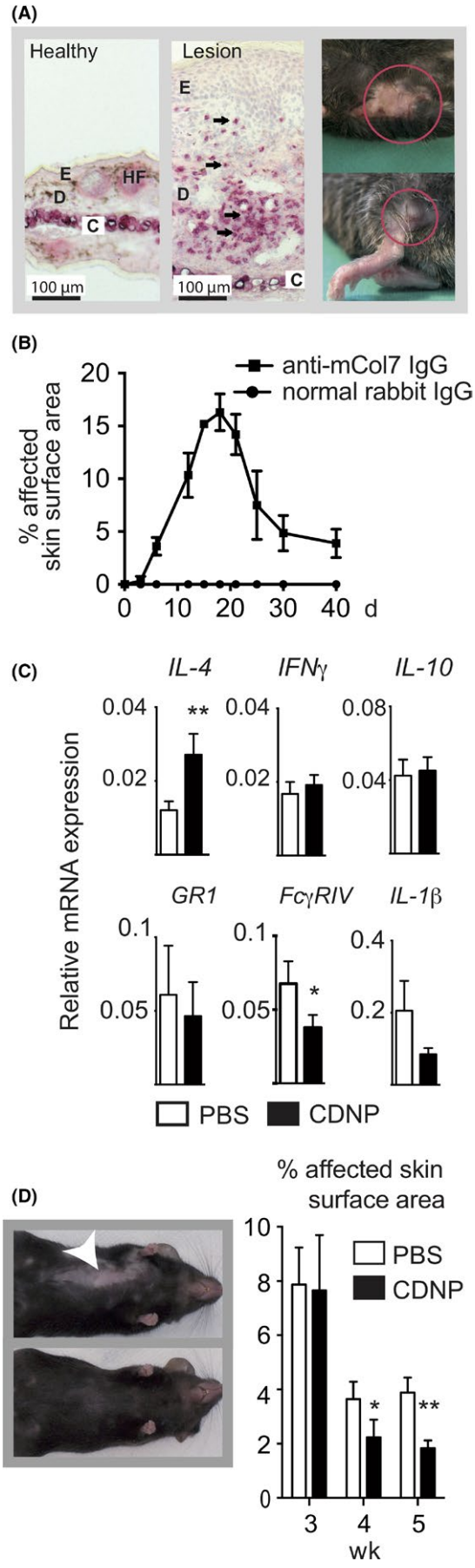

FIGURE 1 CDNP treatment enhances Th2 in lymph nodes and accelerates wound healing. A-D. Induction of skin lesions by passive transfer of rabbit anti-mCol7 IgGs into mice. A, Skin sections stained for anti-GR1 and representative clinical pictures 6 d after first IgG injection are shown. Note: Thickness of ear skin increases dramatically within lesion. B, The extent of affected body surface over time is shown (mean ± SD from 1 of 3 experiments, n = 5-20). C, The mRNA expression of adaptive (IFNγ, IL-4, IL-10) and (E) innate marker genes (GR1, FcγRIV, IL-1β) in inguinal lymph nodes 5 wk after induction of EBA in the indicated groups is shown. Treatment started 16 d after the initial anti-mCol7 IgG injection (mean ± SEM, n = 27-29 pooled from 4 individual experiments). D, Representative photographs of skin wounds (scarfs and bald spots in untreated control groups, 5 wk after initial IgG injection, white arrowhead) and extent of the affected body surface in indicated groups are shown. The mean ± SEM of individual clinical scores from one representative experiment is shown (n = 6). *Significant differences between either the transcript levels in the lymph nodes of CDNP- and PBS-treated mice or clinical scores (**$P \le .01$, *$P \le .05$, Mann-Whitney *U* test). Abbreviations: C: cartilage; D: dermis; E: epidermis; HF: hair follicle

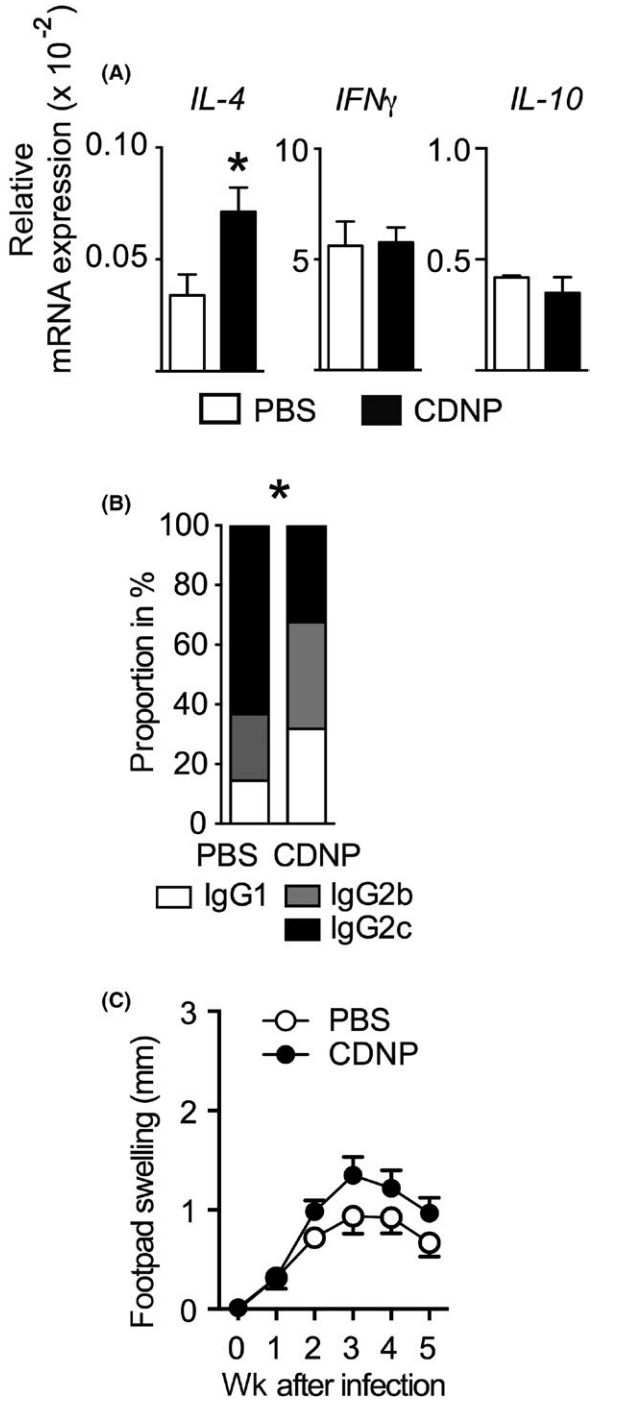

FIGURE 2 CDNPs modulate *L. major* infection towards Th2. A, mRNA expression of IL4, IFNγ and IL-10 in draining lymph nodes and (B) *L. major*-specific IgG1, IgG2b and IgG2c isotypes were determined in serum quantitatively 5 wk after infection with 2×10^6 *L. major* (mean ± SEM, n = 5). C, Footpad thicknesses are shown (mean ± SEM, n = 9 combined from 2 independent experiments with 4-5 C57BL/6 mice). *Significant differences in the mRNA levels and in the IgG1- and IgG2b/c levels between indicated groups are shown (*$P \leq .05$; Mann-Whitney *U* test, n = 5. The data are from one experiment representative of 2 experiments with 5 mice per group)

activated, and (iii) neutrophils infiltrate the damaged site (Figure 1A, B, Fig. S1). The affected surface area increases within 4-17 days and decreases at later time points from approximately 15% at day 20 to 3% at day 40 (Figure 1B). The skin lesions appear mainly as scarfs and bald spots at the end of the observation period of 40 days (data not shown).

Taking advantage of the transient nature of these cutaneous EBA lesions, we treated mice with CDNPs after the mice had developed a clear clinical picture with 10-15% of the skin surface area affected (16 days after initial injection of anti-mCol7-specific IgGs). Due to the fact that inflammatory infiltration ceased 5 weeks after initial IgG injection, the expression of inflammatory cytokine genes could not be detected in the skin (data not shown). However, in inguinal lymph nodes, a significantly increased mRNA expression of the anti-inflammatory Th2 cytokine IL-4 in CDNP-treated groups was detected, whereas the expression of IFNγ and IL-10 was unchanged (Figure 1C). Additional analysis revealed a significantly decreased expression of FcγRIV mRNA in the lymph nodes of CDNP-treated mice (Figure 1C). Moreover, the level of GR1 and IL-1β transcripts in CDNP-treated mice showed a decreasing tendency without reaching significance (Figure 1C). Remarkably, corresponding to the altered cytokine milieu in the lymph nodes, the affected surface area was significantly reduced 2 weeks after treatment with CDNPs, which became even more pronounced 3 weeks after treatment (Figure 1D).

3.2 | CDNPs promote Th2 differentiation in *L. major* infection

To determine whether the induction of Th2 is a general feature of CDNPs, we studied the effect of CDNPs treatment on *L. major* infection in mice, which is a prototypical model for T-cell differentiation into Th1/Th2 cells. *L. major* is an intracellular parasite that induces inflammatory cutaneous lesions at the site of infection. In contrast to EBA lesions, which heal after cessation of anti-mCol 7 IgG binding, *L. major* persists in the skin at the infection site.[13] To detect whether Th2 was induced, resistant C57BL/6 mice were infected with *L. major* and treated with CDNPs, after which the level of IFNγ, IL-4 and IL-10 gene transcription was analysed in the popliteal lymph nodes 5 weeks after infection (Figure 2A). A significant increase in IL-4 expression was observed in CDNP-treated mice (Figure 2A). To validate this increase in Th2 immunity at a functional level IgG1, 2b and 2c, isotypes were analysed and compared quantitatively. IL-4 supports production of IgG1, whereas IFNγ the complement-activating isotypes IgG2b and IgG2c.[14] In accordance with increased IL-4 transcripts, a significantly higher level of the *L. major*-specific IgG1 isotype (Figure 2B) was observed. Even though IgG1 lacks the capacity to kill *L. major* parasites by complement activation, the footpad swelling was only slightly affected without reaching significance (Figure 2C). These data suggest that CDNP treatment does not convert resistance to *L. major* infection towards susceptibility but mildly modulates disease progression.

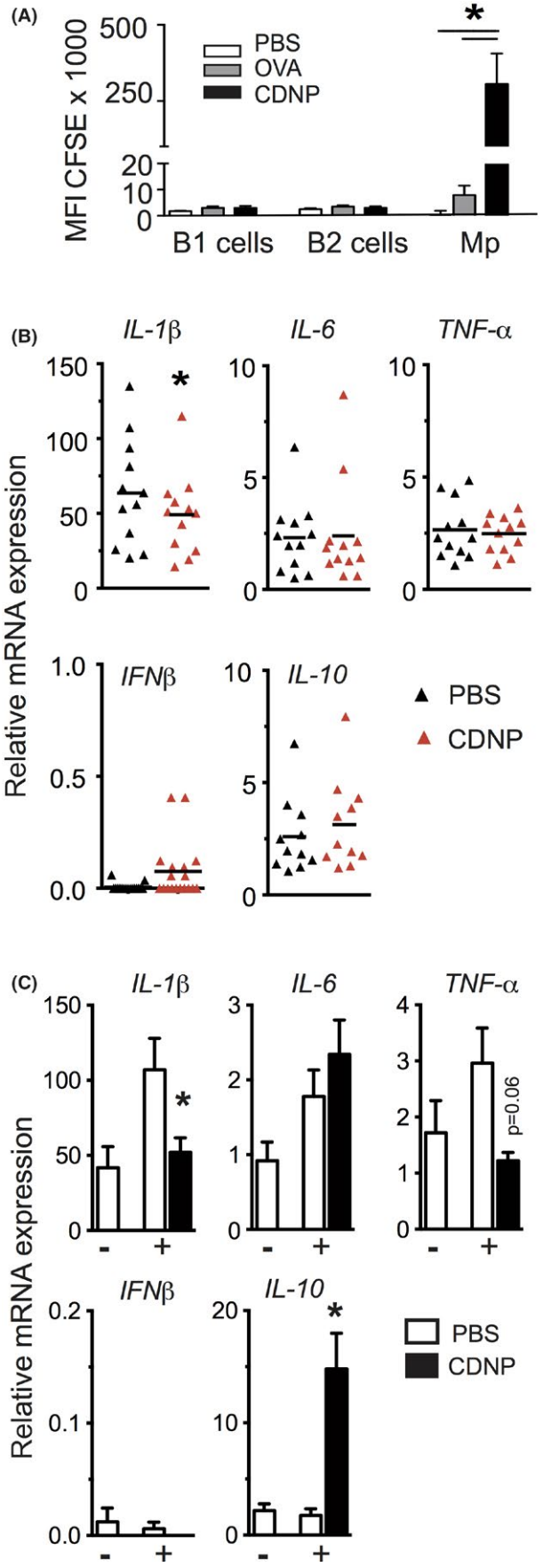

FIGURE 3 CDNPs modulate TLR signalling. A, Uptake of CFSE-labelled CDNPs or ovalbumin (OVA) was analysed in peritoneal B1 cells ($CD11b^{med}$, $B220^{med}$), B2 cells (SSC^{low}, $B220^{hi}$) and macrophages ($CD11b^{hi}$, $F4/80^{+}$). Gating strategy and histograms are shown in Fig. S2. B, IL-1β, IL-6, TNF-α, IFNβ and IL-10 mRNA levels were analysed in peritoneal macrophages after 2 h of culture. Each triangle represents one mouse (*$P \leq .05$ vs PBS; Wilcoxon signed rank test; n = 12 from 3 independent experiments). C, Peritoneal macrophages were cultured with zymosan (+) or without zymosan (−), and CDNPs or PBS were added. Cells were harvested 2 h later, and mRNA expression levels were determined. The data (mean ± SEM) were obtained from 4 independent experiments with n = 1-2 (*$P \leq .05$; one-way repeated-measures ANOVA with Newman-Keuls post hoc test for comparing 3 groups and Wilcoxon signed rank test for comparing 2 groups)

3.3 | CDNPs decrease the expression of pro-inflammatory cytokine genes in peritoneal macrophages

Our data clearly show that porcine CDNPs modify immune responses in cutaneous inflammations towards Th2. This indicates that CDNPs must be taken up by APC and presented to T cells. To test this, CDNPs were labelled with CFSE and incubated with murine peritoneal cells. As shown in Figure 3A and Fig. S2, $CD11b^{hi}$/F4/80+ macrophages had highly efficient uptake of CDNPs. The increase in CFSE was approximately 100-fold higher compared to B1 and B2 cells. Analysis of the gene expression levels of IL-1β, IL-6, TNF-α, IFNβ and IL-10 revealed no increase in expression of cytokine transcripts. Instead, the basal level of IL-1β transcripts decreased significantly in cultures containing CDNPs (Figure 3B). Thus, CDNPs alone do not trigger inflammation via pattern recognition receptors. To assess whether CDNPs modulate functions of activated cells, peritoneal macrophages were stimulated with the yeast cell wall component zymosan to induce pro-inflammatory responses (Figure 3C). Zymosan treatment increased expression of IL-1β, which was decreased approximately twofold by adding CDNPs to the culture media. The number of IFNβ and IL-6 transcripts was constant, whereas that of TNF-α decreased 2.4-fold, almost reaching significance. Moreover, addition of CDNPs to the culture media increased the expression of IL-10 approximately 10-fold. In conclusion, CDNPs induced the expression of the regulatory cytokine IL-10 and did not support the expression of pro-inflammatory cytokine genes.

3.4 | CDNPs improve wound re-epithelialization in human skin tissue cultures

CDNPs modulate ongoing T-cell responses towards Th2 immunity in mice, which suggests that CDNPs may act primarily in T-cell compartments of lymphoid organs. Additionally, our results indicate that CDNPs promote an anti-inflammatory phenotype in cultured murine macrophages. We next asked whether CDNPs would have any effect on human tissues with the rationale that keratinocytes and

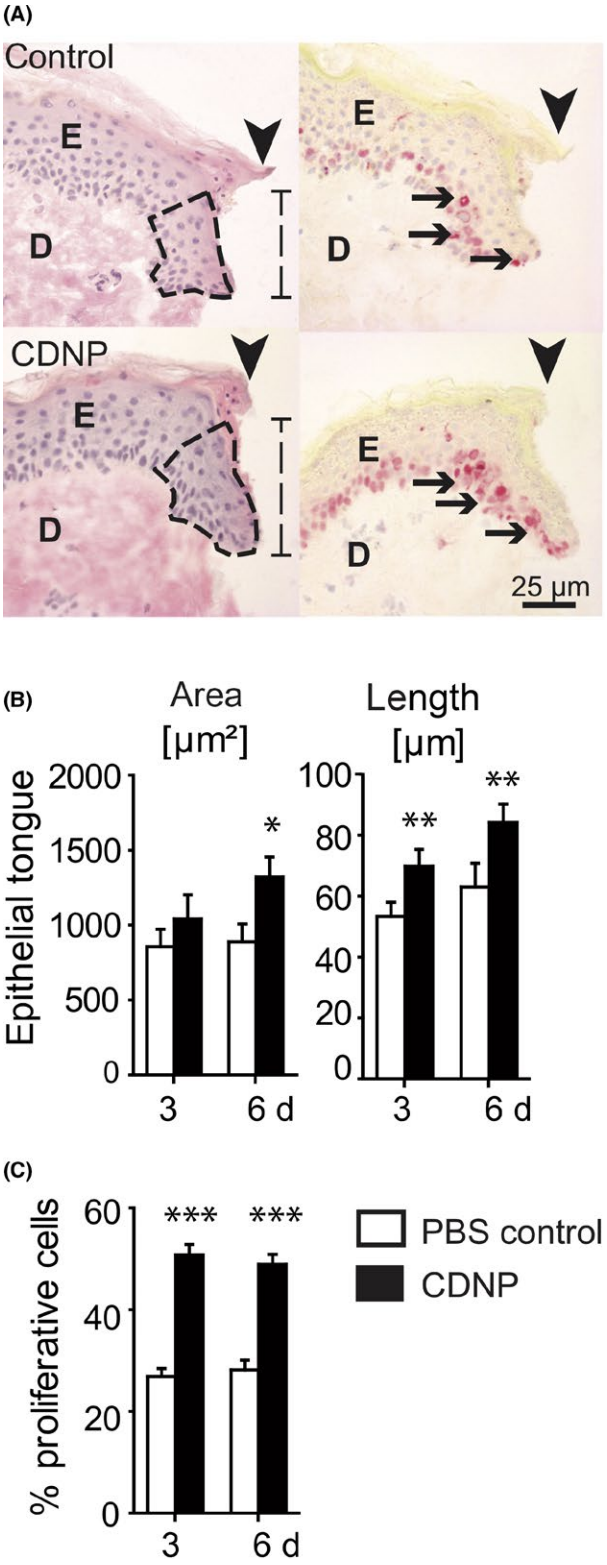

FIGURE 4 CDNPs support growth of human keratinocytes in culture. A "punch-within-a-punch" biopsy was prepared (Fig. S4), and 100 µl CDNPs or PBS was added to the 1 ml culture media. A, Cryosections were prepared after 3 d of culture and stained either with H&E (left panel) or for the proliferation marker anti-Ki-67 (right panel). Arrowheads indicate the edge of the initial punch. B, Growth of epithelial tongues was assessed after 3 and 6 d of culture by ImageJ. C, The percentage of proliferating cells (black arrows) within the newly formed epithelial tongue is presented. (Mean ± SEM, for 4 healthy donors including all inner and outer wound edges per subject) *Significance relative to control data denoted by $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$.) Abbreviations: E: epidermis; D: dermis

skin-resident dendritic cells might respond to CDNPs because both express pattern recognition receptors.[15,16] Human skin biopsies, which were obtained from aesthetic surgeries, were punched twice to obtain 2 inner and 2 outer edges and cultured for 6 days (Fig. S4). Either 3 or 6 days after culture of these "punch-within-a-punch biopsies," the newly formed epithelial tongue was measured. Interestingly, the addition of CDNPs to the culture media significantly increased the length and area of the new epithelial tongue (Figure 4A,B). Staining for the proliferation marker anti-Ki67 revealed that CDNPs stimulated the proliferation of basal keratinocytes (Figure 4C).

4 | DISCUSSION

Chronic cutaneous inflammation is especially common in people with immune diseases and is characterized by persistent pro-inflammatory cells in lesions, thus generating pro-inflammatory cytokines (e.g, IL-1, TNF-α and IFNγ).[17] Because treatment options are limited and hampered by undesirable side effects, there is an urgent need to identify new innovative therapeutic approaches.[18] To address these limitations, we tested whether porcine CDNPs could improve cutaneous inflammation. Previous studies have shown that CDNPs have therapeutic capacities and are able to modulate the expression of cell adhesion molecules such as VCAM and ICAM in vitro.[1,4,19] CDNPs are particles composed of proteins and traces of nucleic acids.[2] Treatment with detergent had no impact on diameter and concentration of CDNPs in contrast to treated rough microsomes (RM) derived from the endoplasmic reticulum (Fig. S3A). This indicates that CDNPs are stable particulate protein-nucleic acid complexes that are not surrounded by a lipid bilayer and can be clearly distinguished from vesicular structures such as microsomes, exosomes, microparticles and apoptotic bodies. Earlier transmission electron microscopic studies demonstrated that CDNPs appear as spherical structures with diameters between 50 and 100 nm.[19] Using scanning electron microscopy, diameters between 100 and 200 nm were observed (Fig. S3). Measurements of CDNPs with 10^{10} particles/ml with NanoSight LM10 revealed particle sizes between 115 and 200 nm (Fig. S3).

Here, we investigated the effects of CDNPs on cutaneous inflammation. Our results clearly show that injection of CDNPs induces Th2 immunity in two cutaneous inflammation models (Figures 1 and 2). The question now becomes how CDNPs induce Th2 immunity. One possibility is that the xenogeneic peptides derived from CDNPs are presented to murine T-cell receptors, which leads to activation of CD4+ T cells and differentiation towards Th2. CD4+ T-cell differentiation into Th2 cells is induced by (i) the presence of IL-4 at the time point of T-cell activation, (ii) weak T-cell receptor signalling strength due to the lack of costimulatory pro-inflammatory signals or (iii) low-avidity binding between peptide: MHC class II and T-cell receptors.[20]

Basophils and mast cells are described as the major cells that provide a Th2-inducing environment by the secretion of IL-4. However, a role of activated basophils or mast cells in CDNP treatment can be excluded because activation of these cells is associated with oedema formation, redness and leucocyte recruitment at the site of injection.[21] These signs of inflammation have never been observed at the cutaneous side of CDNP injection (data not shown). Therefore, it is likely that Th2 immunity emerges because CDNPs induce a weak T-cell receptor signalling strength without strong pro-inflammatory costimulation. This mechanism has been suggested before for soluble extracellular proteins.[22,23]

The process by which Th2 immunity in lymph nodes supports wound healing at individual distant skin lesions is poorly understood. One possibility is that IL-4 induces the formation of type 2 macrophages.[24] The reduced expression of FcγRIV, which is the IgG receptor on phagocytes and which is crucial for tissue destruction and lesion formation in EBA (Figure 2B), supports the idea that macrophages change functionally to an anti-inflammatory phenotype.[25] In addition, our in vitro data show that CDNPs change the expression levels of pro-inflammatory cytokines such as IL-1β towards more anti-inflammatory cytokines such as IL-10 in macrophages in vitro (Figure 3). In the *L. major* model, CDNPs did not contribute to lesion resolution. But, interestingly, CDNP treatment did not exacerbate lesion size and could be therefore a safe therapeutic option even for infected non-sterile wounds.

In addition to Th2 induction, CDNPs support the growth of keratinocytes in human skin cultures (Figure 4); basal keratinocytes especially showed stronger proliferation after adding CDNPs to the culture media (Figure 4D). One possible explanation for this could be that CDNPs stimulated the expression of cytokines and growth factors such as EFG and FGF family members, which induce keratinocyte proliferation.[26]

In conclusion, our data demonstrate that CDNPs have therapeutic capacities on inflammatory cutaneous conditions. It will be important in further studies to identify the active components of CDNPs and to determine the underlying mechanisms. Initial studies suggest a crucial role for Annexin V.[3] In addition, it will be interesting to find out whether allogeneic CDNPs would have similar effects as it has been indicated in initial studies of sepsis.[3] We believe that this new approach is valuable and might provide new therapeutic options in modulating mild immune reactions with minimal side effects to help balance chronic disease conditions such as non-healing wounds.

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AUTHOR CONTRIBUTIONS

N.K. and E.H. designed and performed experiments; S.M., KU.K. and M.B. did research; C.C.C. contributed to NTA analysis and to manuscript writing; F.H. and L.H. isolated CDNPs; MK did SEM analysis; R.J.L. contributed with material and did data analysis; S.S. contributed with material and expertise for human skin cultures; J.W. designed the study and contributed to manuscript writing; K.K. directed the study and wrote the manuscript; and all authors read and approved the final manuscript.

CONFLICT OF INTEREST

Patents for the in vivo use of CDNPs have been applied for by Varicula Biotec GmbH, and KU.K. is listed as an inventor on these applications. Varicula Biotec GmbH partially funded this study but had no influence on the study design, interpretation of data or the final form of the manuscript. F.H., L.H. and KU.K. are shareholders in Varicula Biotec GmbH. K.K. and KU.K. are part-time scientific consultants for Varicula Biotec GmbH.

ORCID

Ralf J. Ludwig http://orcid.org/0000-0002-1394-1737
Kathrin Kalies http://orcid.org/0000-0002-8929-4249

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

FIGURE S1 Induction of skin lesions by passive transfer of rabbit anti-mCol7 IgGs into mice. (A) GR1, FcγRIV and IL-1β transcript levels in non-lesional (−) and lesional (+) skin are depicted. (B) IgG and C3 deposition at the DEJ 6 and 40 days after initial anti-mCol7 or normal rabbit (NR) IgG injection and (C) their quantitative assessments are shown. *Significant differences between non-lesional and lesional skin (*$P \leq .05$, **$P \leq .01$,***$P \leq .001$, ****$P \leq .0001$, Mann-Whitney U-test, n = 4-13). Abbreviations: D, dermis; E, epidermis.

FIGURE S2 Gating strategy for identification of peritoneal B1 and B2 cells, Macrophages (Mp) and CDNP ingestion. (A) Cells were harvested and stained for B220, CD11b and F4/80 surface expression and analyzed by flow cytometry. B1 cells were considered CD11bmed, B220med; B2 cells (SSClow, B220hi) and macrophages (CD11bhi, F4/80+). (B) As control for active uptake the CFSE-MFIs of internalized CDNPs incubated at 4°C was substracted from those incubated at 37°C. A representative histogram of CDNP ingestion by macrophages is shown. (C) Representive histograms for ingestion of CFSE-labelled CDNP or Ovalbumin by B1, B2 and Macrophages is shown.

FIGURE S3 CDNPs are submicron particles. (A) CDNPs at a protein concentration of 150 μg/ml were diluted $1:10^3$ in either PBS alone or in 0.1% Triton X-100 (left panel). ER-derived RM (right panel) were diluted 1:104 in either PBS (black line) or 0.1% Triton X-100 (dotted line). The samples were subsequently analyzed by NTA using a NanoSight LM10. (B) Representative scanning electron microscope images of CDNPs (arrowheads) are shown.

FIGURE S4 Human skin biopsies, which were obtained from aesthetic surgeries, were punched twice to obtain 2 inner and 2 outer edges. An H&E stain of a "punch-within-a-punch" biopsy is shown. Arrowheads indicate the 4 wound edges.

TABLE S1 Primer sequences, amplicon sizes, and gene accession numbers of the analyzed genes[a]

APPENDIX S1 Supplementary Materials and Methods.

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