Highly Specific Auto-Antibodies against Claudin-18 Isoform 2 Induced by a Chimeric HBcAg Virus-Like Particle Vaccine Kill Tumor Cells and Inhibit the Growth of Lung Metastases

Thorsten Klamp1, Jens Schumacher2, Georg Huber1,5, Christoph Kühne2, Ulrich Meissner2, Abderraouf Selmi1, Thomas Hiller1, Sebastian Kreiter3, Jürgen Markl2, Özlem Türeci4, and Ugur Sahin1,3

Abstract

Strategies for antibody-mediated cancer immunotherapy, such as active immunization with virus-like particle (VLP)-based vaccines, are gaining increasing attention. We developed chimeric hepatitis B virus core antigen (HBcAg)-VLPs that display a surface epitope of the highly selective tumor-associated cell lineage marker claudin-18 isoform 2 (CLDN18.2) flanked by a mobility-increasing linker. Auto-antibodies elicited by immunization with these chimeric HBcAg-VLPs in 2 relevant species (mouse and rabbit) bind with high precision to native CLDN18.2 at physiologic densities on the surface of living cells but not to the corresponding epitope of the CLDN18.1 splice variant that differs by merely one amino acid. The induced auto-antibodies are capable of efficiently killing CLDN18.2 expressing cells in vitro by complement-dependent and antibody-dependent cell-mediated cytotoxicity. Moreover, they provide partial protective immunity against the challenge of mice with syngeneic tumor cells stably expressing CLDN18.2. Our study provides a first proof-of-concept that immunization combining VLPs as antigen carriers with specific conformational epitopes of a highly selective differentiation antigen may elicit auto-antibodies with high cytoidal and tumoricidal potential. Cancer Res; 71(2); 12–8. ©2011 AACR.

Introduction

Monoclonal antibodies (mAb) against tumor-associated surface antigens have emerged as promising therapeutics in oncology (1), which execute their antitumoral activity via several independent modes of action. Among these, antibody-dependent cell-mediated (ADCC) and complement-dependent cytotoxicity (CDC) are considered the most relevant ones (2, 3). IgG FcγR polymorphisms were revealed to be independent predictors of clinical outcome in patients treated with the antibody drugs cetuximab, trastuzumab, and rituximab (4–6), and polymorphisms in the complement factor C1qA correlate with prolonged response in follicular lymphoma patients following rituximab therapy (7), further supporting the clinical relevance of CDC and ADCC.

The success of recombinant tumor-targeting mAbs (8, 9) has boosted various innovative antibody-based approaches. Among these, the active induction of auto-antibodies to cancer-associated self-antigens has several theoretical advantages over passively administered mAbs (10). Although mAbs are eliminated from the blood with half-lives ranging from days to weeks, the titer of vaccine-induced antibodies may persist for years. This and the fact that the cost of goods sold of recombinant vaccines is much lower compared with the cost of mAbs (11) is of key importance, as, besides cost considerations, there is no rationale for stopping the clinically effective administration of a recombinant antibody.

A prerequisite for the induction of specific auto-antibodies against self-proteins is to break B-cell tolerance. This can be accomplished by immunization with chimeric virus-like particles (VLP; ref. 12), as the highly repetitive, dense display of the inserted epitopes seems optimal for B-cell receptor cross-linking (13). One of the best characterized capsid proteins is the hepatitis B virus core antigen (HBcAg), which assembles spontaneously to particulate icosahedral nucleocapsids (14). The cloning of foreign epitopes into the major immunodominant region (MIR) at the tip of the VLP spike confers high immunogenicity to the inserted heterologous sequence (15). Originally, VLPs were equipped with pathogen epitopes and were assessed as vaccines against infections (16). Later, self-antigen–derived epitopes, including those from cancer-associated molecules, were engineered into the HBcAg backbone (12). CTL responses as well as auto-antibodies have been elicited by VLP immunization (12). The capability of such auto-antibodies to mediate cell killing via immune effector mechanisms and to

Authors’ Affiliations: 1Department of Internal Medicine III, Translational and Experimental Oncology, and 2Institute of Zoology, Johannes Gutenberg-University, Obere Zahlbacherstr. 63, 55131 Mainz, Germany; 3TRON, Center of Translational Oncology and Immunology; and 4Ganymed Pharmaceuticals AG, Mainz, Germany; and 5Section for Virology, Medical University Innsbruck, Innsbruck, Austria

Corresponding Author: Ugur Sahin, Department of Internal Medicine III, Translational and Experimental Oncology, Johannes Gutenberg-University, Obere Zahlbacherstr. 63, 55131 Mainz, Germany; Phone: 49-06131-17-8055; Fax: 49-06131-17-8054; E-mail: sahin@uni-mainz.de

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confer protection against tumor challenge, however, has so far not been shown.

The objective of our study was to evaluate VLP-induced auto-antibodies for their capability to mediate ADCC or CDC and to provide a first proof-of-concept that prophylactic vaccination with chimeric VLPs protects mice against challenge with syngeneic tumor cells.

As a model antigen, we chose isoform 2 of the tight junction molecule claudin-18 (CLDN18.2), which is associated with gastroesophageal, pancreatic, and other cancers (16, 17). CLDN18.2 is a highly selective cell-lineage marker and confined to short-lived differentiated epithelia of the gastric mucosa; it is absent from the gastric stem cell zone and any other healthy tissue (17). A recombinant mAb against this target is currently in clinical Phase II trials, which makes the evaluation of CLDN18.2 as a target for active immunization therapy particularly interesting.

Materials and Methods

Animals

Mice were obtained from the Laboratory Animal Facility, Johannes Gutenberg-University (Mainz, Germany), and rabbits were obtained from Charles River Laboratories. All animals were maintained under specific pathogen-free conditions.

Cell lines

The human gastric adenocarcinoma cell line NUGC-4, endogenously expressing CLDN18.2, was obtained from JCRB. HEK293 and CHO-K1 cells were obtained from ATCC and endogenously expressing CLDN18.2, was obtained from JCRB. Murine CT26 colon carcinoma cells from ATCC were stably transfected with CLDN18-encoding plasmids. Murine mAb 179 (IgG2a, murine) is cross-reactive with both CLDN18 isoforms (17).

Chimeric HBcAg-CLDN18.2-VLPs

The HBcAgΔ gene encodes a C-terminally truncated protein (amino acid 1–150), which lacks the nucleotide binding domain and terminates with a short spacer sequence linked to a hexahistidine-tag. This gene was codon-optimized for expression in Escherichia coli modified to contain unique cloning restriction sites, and cloned into plasmid pET21a (Merck). Two HBcAg-CLDN18.2 chimeras were derived from this backbone: CLDN-VLPs display the CLDN18.232–41 peptide (TQDLYNNPVT) in the MIR (18) replacing Pro-79 and Ala-80 of HBcAgΔ, and CLDN-Link-VLPs feature this CLDN18.2 epitope, flanked on both sides by glycine-rich linkers (G4SG4). All 3 HBcAg constructs contain a C-terminal hexahistidine-tag for purification (19) and for optimized assembly (20) and were expressed in the E. coli strain BL21 DE3 (Merck). For IPTG-induced large-scale expression, an inoculum was diluted in 2-L LB medium and incubated at 30°C for 8 to 12 hours. After cell harvest, lysis, and debris removal, VLPs were purified under mild denaturing conditions, reassembled in vitro (19), and enriched by density gradient ultracentrifugation. Pooled VLP-containing fractions were dialyzed against PBS. The purity and integrity was analyzed by SDS-PAGE, nondenaturing agarose gels, and electron microscopy as described previously (21, 22).

Electron microscopy and three-dimensional protein reconstruction

Purified HBcAg-VLPs were adsorbed onto glow-discharged carbon-coated copper grids, negatively stained with 0.2% (w/v) uranyl acetate and analyzed with a Tecnai-12 electron microscope (FEI-Company) at a magnification of 68,000-fold. The 3D-reconstructions of chimeric HBcAg-CLDN18.2-VLPs with icosahedral triangulation numbers of $T = 4$ were based on cryo-electron micrographs taken with a Tecnai-F20 microscope. Image analysis and 3D reconstruction was done as previously (23) with the Imagej software package (Image Science); ~1,400 single particles from each VLP species were applied. Homology modeling with the X-ray structure of HBcAg-VLPs (24) as a template and modified HBcAg-sequences as targets was done in MODELLER-9v3 (http://salilab.org/modeller/). Rigid-body and flexible fitting of the obtained molecular models into the cryo-EM structures were conducted with the UCSF-Chimera software (http://www.cgl.ucsf.edu/chimera/) and NMFF (http://mmts.org/software/nmff.html).

Immunization of animals, auto-antibody purification, and control antibodies

Immunizations were done with purified HBcAg-VLPs in PBS (Table 1); some of these were done with adjuvants AblSCO-100 (Isonova), Montanide ISA720 (Seppic), or CFA/IFA (Sigma-Aldrich). Sera from immunized animals were collected 1 week after each immunization and processed with the Melon Gel IgG purification kit (Thermo Scientific).

Control mAbs ch-175D10 (IgG1, chimeric), its parental mouse mAb mu-175D10 (IgG1, murine), and 163E12 (IgG3, murine) recognize conformational epitopes in the first extra cellular domain of CLDN18.2 but not of CLDN18.1 and, except for mu-175D10, exhibit strong target-specific CDC and ADCC. mAb 179 (IgG2a, murine) is cross-reactive with both CLDN18 isoforms (17).

ELISA assays

Microplates were coated with 500-ng antigen per well [BSA-conjugated CLDN18.232–41 peptide (JPT Peptide Technologies) or HBcAgΔ-VLPs], blocked, washed, and incubated for 1 hour at room temperature with serially 10-fold diluted, unpurified mouse or rabbit antisera in triplicates. Bound antibodies were detected by species-specific, HRP-conjugated secondary anti-IgG antibodies (Jackson ImmunoResearch), followed by incubation with Turbo TMB (Thermo Fisher Scientific). Absorbance at 450 nm was measured using a fluorescence plate reader (Perkin Elmer). The endpoint ELISA antibody titer was calculated as the highest dilution of antisemum, specifying an OD450 nm twice the mean, plus 3 SDs of premimmune sera at the same dilution.

Flow cytometry-based assays

Antibody binding to native epitopes was analyzed by FACS with CLDN18-transfected HEK293 cells or NUGC-4 cells. Cells were washed and incubated with 1:50 diluted, Melon gel-purified, polyclonal mouse or rabbit IgGs or control mAbs (50 μg/mL) for 30 minutes at 4°C. Primary antibodies were detected by staining with Alexa Fluor 647-conjugated goat
anti-mouse or anti-rabbit IgG secondary antibodies (Invitrogen). Living cell counts were plotted against the fluorescence intensity of cells in the Alexa647 channel using FACSCalibur equipment (BD Biosciences).

Immunofluorescence microscopy
CHO-K1 cells were transiently transfected with plasmids encoding CLDN18 orthologues from rabbits or mice, cultured for 24 hours on cover slides, washed, and stained under native conditions with the respective antibody. The cells were fixated in 100% methanol at −20°C, air-dried, and incubated with secondary antibodies coupled to Cy3-fluorescent dyes (Jackson ImmunoResearch) and with Hoechst 33342 (Invitrogen). Slides were washed, mounted in fluorescence mounting medium, and images were taken with a Leica DMR fluorescence microscope (Leica Microsystems).

Complement-dependent cytotoxicity assay
CHO-K1 target cells stably transfected with human CLDN18 variants were transiently transfected with luciferase encoding in vitro–transcribed RNA, seeded in 96-well tissue culture plates, and incubated overnight at 37°C. The 1:10 diluted, polyclonal anti sera from immunized animals were added to the cells in triplicate wells per serum sample and incubated for 30 minutes at 37°C. Heat-inactivated or active human serum, as a complement source, and D-Luciferin (BD Biosciences) containing reaction buffer were added, and luminescence signals were measured with an Infinite 200 Reader (Tecan). Cytolytic activity was calculated as follows:

\[
\% \text{Cell lysis} = \frac{100\% - \left( \frac{\text{Signal antisera} - \text{Signal } 100\% \text{ killing}}{\text{Signal untreated cells}} \right)}{100} \]

Table 1. Overview of performed immunization experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Group/Animal no.</th>
<th>Immunogen</th>
<th>Adjuvant</th>
<th>Route</th>
<th>Amount</th>
<th>linear epitope &amp; native epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1</td>
<td>w/o</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>4/4</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>w/o</td>
<td>i.v.</td>
<td>2 × 50 μg</td>
<td>5/5</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>2 × 50 μg</td>
<td>4/5</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>CLDN-Link-VLPs</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ISA720</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>3/5</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Abisco</td>
<td>s.c.</td>
<td>4 × 100 μg</td>
<td>5/5</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>w/o</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>0/5</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>CLDN-VLPs</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>ISA720</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>2/4</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>KLH-conjugated</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>CLDN18.2</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>2 × 50 μg</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>peptide</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>ISA720</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>2/3</td>
<td>CLDN18.2</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Degraded CLDN-Link-VLPs</td>
<td>CFA/IFA</td>
<td>s.c.</td>
<td>3 × 50 μg</td>
<td>0/5</td>
</tr>
</tbody>
</table>

| Rabbit  | 1               | HBcAgΔ-VLPs | w/o | i.d. | 4 × 100 μg | –     | –                             |
|         | 2               | CFA/IFA    | s.c. | 4 × 100 μg | –     | –                             |
|         | 3               | CLDN-Link-VLPs | w/o | i.d. | 4 × 100 μg | –     | –                             |
|         | 4               | CFA/IFA    | s.c. | 4 × 100 μg | +     | +                             |
|         | 5               | CFA/IFA    | s.c. | 3 × 200 μg | +     | +                             |
|         | 6               | ISA720     | s.c. | 3 × 200 μg | +     | +                             |
|         | 7               | CFA/IFA    | s.c. | 3 × 200 μg | +     | +                             |
|         | 8               | ISA720     | s.c. | 3 × 200 μg | +     | +                             |

Groups of mice consisted of 5 animals, except for groups 1 and 9 (4 animals) and groups 10, 12, and 13 (3 animals).
Immunizations were performed without adjuvants (w/o) or with CFA for primary and IFA for booster immunizations. Alternatively, Montanide ISA720 (ISA720) or AbISCO-100 (Abisco) was used.
The number of mice per group displaying reactivity against the linear BSA-conjugated CLDN18.232–41 peptide was determined by ELISA. + and − symbols indicate the reactivity of rabbit sera. Animals with endpoint titers lower than −log10=2.875 were classified as negative.
The number of mice per group displaying reactivity against CLDN18.2 in its native conformation on the surface of living cells as determined by IF or FACS. + and − symbols indicate the reactivity of rabbit sera.
Antibody-dependent cellular cytotoxicity assay

NUGC-4 target cells were transiently transfected with luciferase encoding RNA, seeded in 96-well tissue culture plates, and incubated overnight at 37°C. Plates were incubated with purified polyclonal IgG from the sera of immunized animals. Ficoll-Paque-purified human PBMCs were added as effector cells (effector to target ratio = 10:1) and incubated for 5 hours, followed by the addition of D-Luciferin containing reaction buffer. Cytolytic activity was determined as described above. Cells incubated only with antibodies, untreated, and complement served as a reference.

Assessment of prophylactic vaccination efficacy in vivo

BALB/c mice were vaccinated 3 times (day 1, day 14, and day 28) with 50 μg HBcAgΔ-s, CLDN-Link-VLPs, or PBS as control, all formulated in AbISCO-100 (Isconova). Two weeks after the last immunization, 1 x 10^5 syngeneic CT26 colon cancer cells stably expressing murine CLDN18.2 were administered into the tail vein. Thirteen days later, the mice were sacrificed and their lungs were weighed and subjected to microscopic analysis to assess the load of pulmonary metastases. Statistical analysis of lung weights was done by ANOVA, followed by Dunn’s test. For histopathologic assessment, 3-μm thick sections of formalin-fixed and paraffin-embedded lungs were deparaffinized and rehydrated, followed by heat-induced epitope retrieval in a citrate buffer at pH 6. After the quenching of endogenous peroxidases by H2O2, unspecific antibody binding sites were blocked with 10% goat serum, followed by overnight incubation with polyclonal rabbit antibody binding sites were blocked with 10% goat serum, followed by overnight incubation with polyclonal rabbit antibody, anti-CLDN18 (Mid) (Invitrogen) at 4°C, and normal tissue areas were found using ImageJ Software v.1.44. Statistical differences between groups were assessed by ANOVA, followed by Dunn’s test.

Results

Design of chimeric HBcAg-CLDN18.2-VLPs for the induction of CLDN18.2 specific antibodies against orthologues from 3 species

Our objective was to design a VLP-based vaccine (i) for the induction of auto-antibodies with specificity against the native CLDN18.2 protein, (ii) with potent cytoidal effector functions and (iii) that was suitable for valid proof-of-concept studies in animal models. These specifications imposed several requirements on the epitope to be selected. First, cross-reactivity of antibodies with the highly related splice variant 1 of CLDN18, which is expressed in healthy lung tissue, had to be prevented to avoid harm to this toxicity-relevant organ (17, 25). Both splice variants of the tetraspanin CLDN18 vary in their first extracellular domain (8 amino acids of 51 differ between both isoforms; Fig. 1A); however, the second extracellular loop is identical in both isoforms and, thus, is not suitable for discrimination. Second, the localization of the targeted epitope close to the cell membrane was regarded as beneficial for the Fc-mediated cytolytic activity of induced antibodies (26, 27). Third, biophysical and biochemical properties had to facilitate VLP assembly (28). Fourth, since we wanted to evaluate these VLPs in mice and rabbits as proof-of-concept for subsequent clinical testing in humans, the epitope had to be conserved across these species.

All criteria were satisfied by the CLDN18.232–41 peptide (TQDLYNNPVT) derived from the first extracellular loop of CLDN18.2. Most importantly, this peptide differed from the corresponding CLDN18.1 epitope by a single amino acid (Fig. 1A). The sequence was cloned into the MIR of the HBcAgΔ carrier molecule either directly (resulting in CLDN-VLPs) or flanked by flexible glycine-rich linkers (yielding CLDN-Link-VLPs; Fig. 1B).

Generation and characterization of HBcAg-CLDN18.2-VLPs fulfilling integrity and quality criteria

Purified chimeric and HBcAgΔ-VLPs were found to migrate as distinct sharp bands in native agarose gel electrophoresis (Fig. 2A), indicating the particulate nature of the proteins. Ethidium bromide staining of native agarose gels did not reveal the incorporation of nucleic acids into the VLPs (data not shown).

Negative-staining TEM confirmed VLP formation and the integrity of all constructs. Moreover, it showed an almost uniform size distribution of chimeric HBcAg-CLDN18.2-VLPs, which had a capsid diameter of ~35 nm and were morphologically indistinguishable from HBcAgΔ-VLPs (Fig. 2B). The 3D reconstructions were processed from cryo-electron micrographs of chimeric VLPs. Into these 3D volumes, we conducted rigid-body fitting of the HBcAgΔ-VLP X-ray structure and revealed additional masses at the tips of their capsid spikes (Fig. 2C). Homology modeling and flexible fitting showed that these extra masses on the tip of CLDN-VLPs corresponded to the theoretical volume of the inserted CLDN18.232–41 peptide. The calculated loop structure of the inserted epitope was further supported by a Ramachandran plot (data not shown). Extra masses on the tip of the CLDN-Link-VLP spikes, in contrast, displayed a volume too small for the CLDN18.232–41 epitope plus flanking linkers. Modeling of the entire insert (close-up in Fig. 2C) indicated that the apical loop region of CLDN-Link-VLPs exhibits a high morphologic flexibility. The failure of the applied computational reconstruction method to resolve the structure by picture averaging resulted in a blurring of this region.

In summary, these data imply that the intrinsic assembly capacity of the HBcAg backbone is not hampered by the inserted CLDN18.2 epitope and that flanking linkers in CLDN-Link-VLPs provide a high conformational flexibility.

Immunization of mice and rabbits with HBcAg-CLDN18.2-VLPs results in anti-CLDN18.2 antibodies recognizing a linear epitope of this self-protein

The potency of chimeric VLPs to induce antibody responses against the inserted CLDN18.2 epitope was analyzed through the immunization of mice and rabbits. Importantly, the selected epitope and the tissue distribution of the orthologous...
proteins with strict restriction to short-lived gastric cells are conserved in all 3 species (manuscript in preparation).

BALB/c mice were immunized with CLDN-, CLDN-Link-VLPs, or with KLH-conjugated linear CLDN18.232-41 peptide as a control, and antibody reactivity against the linear CLDN18.232-41 peptide or the HBcAg backbone was found by measuring the ELISA endpoint titer (Fig. 3A). Different immunization protocols were applied—varying the adjuvant, administration route, and immunogen amount—each to groups of 3 to 5 mice (Table 1). We observed that sera from mice immunized with CLDN-Link-VLPs displayed a higher specific reactivity against the linear BSA-conjugated CLDN18.232-41 peptide as compared with mice in other groups (Fig. 3A top panel). Interestingly, mice immunized s.c. with CLDN-Link-VLPs without the addition of adjuvant (group 1 in Fig. 3A) revealed the highest mean endpoint titer against the peptide. Immunization with CLDN-VLP or peptide vaccine formulations, in contrast, resulted less frequently in high endpoint titers (nearly all vs. one third; Table 1). All VLPs induced antibodies against the HBcAg backbone with similar endpoint titers (Fig. 3A bottom panel). Heat denaturation of chimeric VLPs abrogated their capability to induce peptide-binding antibodies without compromising the development of antibodies against the backbone.

Also, in rabbits, we accomplished the induction of high-titer IgG responses against the inserted peptide epitope. CLDN-Link-VLPs gave moderately higher endpoint titers as compared with CLDN-VLPs, again without clear benefit from a co-application of adjuvant (Fig. 3B).

Immunization with CLDN-Link-VLPs results in splice-variant-specific auto-antibodies recognizing CLDN18.2 on the surface of endogenously expressing cancer cells

Since antibodies binding to a denatured epitope in a peptide ELISA are not necessarily capable of recognizing the CLDN18.2 protein in its native conformation on living cells, we tested polyclonal IgG purified from the sera of all immunized animals on unfixed target-positive cells by FACS and IF microscopy.

Figure 1. CLDN18.2 epitope selection and design of chimeric HBcAg-CLDN18.2 constructs. A, cross-species sequence alignment of the first extracellular domain (black rectangle) of CLDN18 isoform orthologues and CLDN18.232-41 epitope (gray box) selected for insertion into HBcAg. B, schematic representations of VLP constructs. The nucleotide-binding domain of wild-type HBcAg was replaced by a glycine-glycine-serine linker and a hexahistidine-tag (black box). In chimeric HBcAg-CLDN18.2 constructs, aa 79 and 80 (P, proline; A, alanine) were replaced by the selected CLDN18.232-41 epitope (TQDLYNNPVT; CLDN-VLP) or by the epitope flanked with glycine-rich linkers (amino acid sequence G4SG4; CLDN-Link-VLP).
All sera were assessed and those that recognized not only denatured but also native epitopes on the cell surface of CLDN18.2 positive cells were retrospectively marked gray in Figure 3.

Of the mice vaccinated with CLDN-Link-VLPs (groups 1–6), ~90% were shown to recognize the linear CLDN18.2 epitope in ELISA (Fig. 3A). One third of these were able to bind to the native CLDN18.2 molecule on transfectants as analyzed by FACS (Table 1; see Fig. 4A for selected examples). One third of CLDN-VLP– and two-thirds of CLDN18.232–41 peptide–immunized mice (groups 7–9 and 10–13, respectively) showed reactivity against the linear epitope. However, none of these recognized the native protein displayed on the surface of transfectants (Table 1; Fig. 4A). In rabbits, all the sera of animals vaccinated with CLDN-Link-VLPs were able to recognize the linear epitope as well as the native protein.

Another observation we made in passing by using CLDN18.1 transfectants as a control was the lack of cross-reactivity with this variant. This prompted us to investigate the isoform-specificity of antibodies by IF microscopy. Since we used CHO cells transfected with either the rabbit or mouse orthologues of CLDN18 splice variants in conjunction with immune sera generated in the corresponding species, this experiment also verified that the induced polyclonal IgG responses indeed recognized the adequate orthologue and, thus, were authentic auto-antibodies (Fig. 4B).

Next, we evaluated the capability of induced auto-antibodies to detect the physiologic densities of the CLDN18.2 protein on the surface of endogenously expressing human gastric cancer cells. Purified polyclonal IgGs from all rabbits immunized with chimeric CLDN-Link-VLPs were shown by flow cytometry to bind strongly to NUGC-4 cells (Table 1; Fig. 4C), whereas IgGs from CLDN-VLP or HBcAgΔ-VLP immunized rabbits displayed no or very weak reactivity (data not shown).

In summary, these experiments show that reactivity against the denatured epitope is accomplished by both VLP species; however, CLDN-Link-VLPs were clearly superior in eliciting auto-antibodies that recognize the native protein in physiologic densities on the surface of endogenously expressing tumor cells.
Auto-antibodies against CLDN18.2 induced by immunization with CLDN-Link-VLPs kill target positive cells by CDC and ADCC

A key objective of this study was to induce specific auto-antibodies, capable of lysing cells by Fc-mediated immune effector mechanisms such as ADCC and CDC. Purified IgG from sera, for which we had shown target-specific binding to cell surface–displayed epitopes, were subjected to luciferase-based CDC and ADCC in vitro assays and compared with recombinant mAbs 163E12 and ch-175D10 as positive controls.

Sera from rabbits immunized with CLDN-Link-VLPs lysed CLDN18.2 transfected cells in a complement factor–dependent manner with killing rates of up to \( \frac{C24}{80} \). Most importantly, CLDN18.1 transfected cells were not lysed, and sera from rabbits immunized with CLDN-VLPs or HBcAg\( ^\Delta \)-VLPs had no effect (Fig. 5A top panel). Analogously, exclusively antisera from mice immunized with CLDN-Link-VLPs and previously shown to recognize native CLDN18.2 protein, displayed strong target-specific CDC with lytic activity of up to 55% and was comparable to cell lysis obtained by mAb 163E12 (Fig. 5A bottom panel).

ADCC was analyzed by incubating purified serum IgGs with luciferase expressing NUGC-4 cells as targets and human PBMCs as effectors. IgG derived from rabbits immunized with CLDN-Link-VLPs resulted in cell killing of up to 45% (Fig. 5B), reaching levels observed with mAbs 163E12 and ch-175D10 and known to bind CLDN18.2 with nanomolar affinities. Antibodies from rabbits immunized with HBcAg\( ^\Delta \)-VLPs, in contrast, were only marginally active in ADCC (Fig. 5B). The removal of VLP-backbone reactive antibodies with column–immobilized HBcAg\( ^\Delta \)-VLPs further augmented the ADCC of the respective serum (asterisk in Fig. 5B). Similarly, exclusively IgGs derived from CLDN-Link-VLP–immunized but not from peptide-immunized mice mediated ADCC. However, the overall activity was less profound, which is in line with the notion that several mouse antibody isoforms (particularly the IgG1 isotype antibodies) are known to be very inefficient in the induction of ADCC with human effector cells (29). Accordingly, the murine IgG1 mAb mu-175D10 did only marginally induce ADCC, whereas the murine mAb 163E12 (IgG3 isotype) and a chimerized version of mAb 175D10 were found to be very effective.

In summary, these results proved that CLDN-Link-VLP vaccination elicits auto-antibodies efficiently killing CLDN18.2 expressing cells by CDC and ADCC.

Prophylactic vaccination with CLDN-Link-VLPs confers partial protection in an immunocompetent syngeneic mouse tumor model

To evaluate the prophylactic in vivo efficacy of CLDN-Link-VLPs, we used a syngeneic tumor model in immunocompetent BALB/c mice in which pulmonary metastasis formation was induced by the i.v. application of CT26 colon cancer cells that were stably transduced with murine CLDN18.2. Macroscopic analysis of the lungs derived from mice vaccinated with CLDN-Link-VLPs revealed a smaller number of metastatic nodules as compared with HBcAg\( ^\Delta \)-VLP or PBS control groups (Fig. 6A) and significantly lower lung weights close to those of mice not

\[ \text{OD}_{400} = \frac{2.9}{\log_{10}} \]

Figure 3. Serum antibody reactivity against the linear CLDN18.2\( _{32-41} \) epitope and the HBcAg carrier molecule. A, BALB/c mice (3–5 per group) were immunized with chimeric HBcAg-VLPs and KLH-conjugated CLDN18.2\( _{32-41} \) peptide as positive or heat-degraded CLDN-Link-VLPs (*) as negative control using different immunization protocols (see Table 1). Antibody endpoint titers against CLDN18.2\( _{32-41} \) peptide (top) and against the HBcAg carrier (bottom) were determined by ELISA. Endpoint titers are indicated as reciprocals of the highest dilutions of sera required to yield an OD\(_{400}\) of twice as high plus 3 SDs of preimmune sera at the same dilution. The mean value of each group is indicated by a bar. Endpoint titers lower than \(-\log_{10} = 2.9\) (indicated by a dashed line) were classified as negative.

B, NZW rabbits were subjected to a similar immunization procedure (see Table 1) and the reactivity of sera was determined. Error bars represent the SD of triplicate experiments. Light gray–filled circles/bars indicate sera recognizing the protein expressed on cells in its native (versus only denatured = black circles/bars) conformation (see Fig. 4). Groups or single animals treated without adjuvant are underlined.
Figure 4. Specificities of auto-antibodies to native CLDN18.2 expressed on the surface of living cells. All sera were investigated by FACS or IF for reactivity against the cell surface-displayed native epitope (see Table 1, last column). Selected examples are shown in this figure. A, HEK293 cells stably expressing human CLDN18 isoforms were stained under native conditions with polyclonal IgG purified from the sera of immunized rabbits or mice. The immunogens used for vaccination and identifiers of serum-donating animals (e.g., mouse 1/4 means mouse 4 in group 1) are indicated (see Table 1). B, CHO-K1 cells transiently expressing CLDN18 isoform orthologues from rabbits (oc) or mice (mm) were stained under native conditions with polyclonal IgG purified from the sera of CLDN-Link-VLP–immunized animals and analyzed by IF microscopy (controls, isoform cross-reactive mAb179 to show the functionality of constructs and CLDN18.2 specific mAb ch-175D10). C, binding of sera from immunized rabbits to NUGC-4 cells was found under native conditions by flow cytometry. Controls, prebleed sera and mAb ch-175D10.
challenged with tumor cells (Fig. 6B). Moreover, the percentage of cancerous tissue area per whole lung section as calculated after visualizing CT26-CLDN18.2 pulmonary metastases by IHC-staining for CLDN18.2 was significantly ($P < 0.05$) smaller as compared with mice vaccinated with HBcAgΔ-VLPs or the PBS control group (Fig. 6C and D).

In conclusion, these data showed that prophylactic vaccination with CLDN-Link-VLPs mediates partial protection against highly malignant/tumorigenic CT26-CLDN18.2 cells.

**Discussion**

This report details our efforts to characterize auto-antibodies induced by immunization with chimeric HBcAg-CLDN18.2-VLPs in 2 species, gives novel insights into mechanisms of action that may be triggered by VLP-based vaccines, and presents first *in vivo* efficacy data.

The glycine-rich linker sequence in CLDN-Link-VLPs was originally included to facilitate particle assembly by relieving the internal stress forced on HBcAg monomers by the insertion of the foreign peptide (30). The 3D reconstructions revealed that, in CLDN-VLPs, the insert has a rigid conformation, whereas the linker-flanked epitope of CLDN-Link-VLPs has a higher flexibility. Unexpectedly, auto-antibodies elicited by CLDN-Link-VLPs were more capable than those induced by CLDN-VLPs of binding the epitope not only in its denatured, linear condition but also as native surface epitope on endogenously expressing cells. Most likely, the linker displays the inserted epitope in diverse conformations, including those with high antigenicity or mimicking more authentically the loop naturally displayed on cancer cell surfaces. Thus, chimeric VLPs enable the induction of immune responses against integral membrane proteins with small extracellular loops, which in general is not trivial to accomplish but is of high interest because antibodies generated against epitopes that are close to the plasma membrane induce with a higher probability ADCC and CDC as compared with antibodies against epitopes that are distant to the plasma membrane (31).

Characterization of the auto-antibody response taught us several unexpected lessons. First, we showed the feasibility of inducing high titers of cytocidal auto-antibodies against a self-protein that were able to confer prophylactic antitumor effects in a syngeneic mouse tumor model. Even though the CLDN18.2 epitope was conserved in both animal models, the induction of auto-antibodies does not necessarily relate to the breakage of B-cell tolerance, which is not as stringently controlled as T-cell tolerance (32). Approximately 20% of long-lived mature B-cells are considered to be self-reactive (33). Most of these are directed against cytosolic molecules, whereas membrane-bound proteins induce stringent B-cell tolerance (34). B-cell tolerance against ubiquitous membrane proteins is based on the deletion of autoreactive B-cells (35, 36). Low, abundant, sequestered, or tissue-specific membrane-bound proteins, in contrast, cause either reversible anergy of self-reactive B-cells (37) or the B-cells remain functionally naive (38). CLDN18.2 may fall into the latter category because of its strict cell-lineage specificity, since its privileged localization in the luminal layer of the gastric wall and its multimeric

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**Figure 5.** Auto-antibody mediated CLDN18.2-specific cell killing by CDC and ADCC *in vitro*. A, CHO-K1-cells stably transfected with human CLDN18 isoforms and transiently expressing luciferase were incubated with selected sera from immunized rabbits (top) or mice (bottom) and human serum as a source for complement factors to analyze CDC-mediated lytic activity. The percentage of cell lysis was calculated by subtracting background luminescence and referred to completely lysed cells (positive control, mouse mAb 163E12; negative control, inactivated human serum). B, human NUGC-4 cells transiently transfected with luciferase were used as targets for investigating ADCC. Cells were incubated with IgG purified from the sera of immunized animals and with human PBMCs (effector to target ratio = 10:1) in the presence of luciferin (positive controls, mAb 163E12 and ch-175D10; negative controls, mAb mu-175D10). 5* indicates purified IgG from rabbit #5 additionally adsorbed against immobilized HBcAgΔ-VLPs.
complexing with other tight junction components causes many epitopes to be shielded under physiologic conditions.

Second, a single amino acid difference in the selected CLDN18.2 epitope was sufficient to prevent cross-reactivity with CLDN18 splice variant 1 with potentially harmful effects on lung tissue.

Third, we showed for the first time that VLPs may induce auto-antibodies, which were capable of efficiently killing
target-positive cells by ADCC and CDC. VLP-based vaccines have been primarily used to elicit immunity against infections or to develop treatments against chronic autoimmune diseases (12, 14, 39). In both indications, the objective is to induce auto-antibodies capable of blocking or neutralizing pathogens or auto-reactivity-pertaining self-molecules (39–41). Modes of action required for immunotherapy for nonviral cancer, however, are different. Few studies have used VLPs to induce immunity against cancer-associated self-antigens (42–50). In none of these studies were auto-antibodies with antitumoral mode-of-action reported. Since we cross-species reagents for conducting killing assays (human serum or PBMCs combined with mouse or rabbit antisera), cytotoxic activities might even be underestimated (29). Both modes of action are likely to also be responsible for the observed protective immunity against syngeneic tumor challenge after prophylactic vaccination with CLDN-Link-VLPs.

In summary, this is the first report demonstrating a chimeric VLP-based vaccine that elicits high titers of polyclonal auto-antibodies that are capable of recognizing, with high precision, native epitopes of membrane-bound tumor-associated antigens on cancer cells and eliciting cytotoxic activity. This opens new paths for the development of VLPs for cancer therapy.

Disclosure of Potential Conflicts of Interest
Several authors (U. Sahin, Ö. Türeci, T. Klamp, J. Schumacher, T. Hiller) are inventors on a patent application describing HBcAg CLDN18.2-VLPs for cancer immunotherapy. Ö. Türeci is the CEO/CSO of Ganymed Pharmaceuticals, a company holding past patent applications on CLDN18.2 as a therapeutic target for recombinant antibody therapy of cancer. U. Sahin is the CEO of BioNTech, a company developing VLP-based cancer vaccines. All other authors declare no potential conflict of interest.

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Highly Specific Auto-Antibodies against Claudin-18 Isoform 2 Induced by a Chimeric HBcAg Virus-Like Particle Vaccine Kill Tumor Cells and Inhibit the Growth of Lung Metastases

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