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## Abstract

The cover is based on a transmission electron micrograph of a bone-marrow derived dendritic cell (BMDC) that has internalized particles of modified *Concholepas* hemocyanin (Ox-CCH). The image is taken from the article by Arancibia *et al.* (pp. 688-699) in which the authors show that Ox-CCH and its stabilized form are slowly processed and accumulate in endosomelike structures in BMDCs. The persistence and limited processing of Ox-CCH might therefore explain its remarkable adjuvanticity in mammals and beneficial biomedical properties. The colour of the Ox-CCH crystals has been digitally altered to highlight the natural, deep blue colour of hemocyanin for the cover.

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# Enhanced structural stability of *Concholepas* hemocyanin increases its immunogenicity and maintains its non-specific immunostimulatory effects

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**Hemocyanins, which boost the immune system of mammals, have been used as carrier-adjuvants to promote Ab production against haptens and peptides, as immunostimulants during therapy for bladder carcinoma and as a component in therapeutic vaccines for cancer. These biomedical applications have led to growing interest in obtaining hemocyanins with high immunogenicity. Here, we study the immunological properties of a modified oxidized *Concholepas concholepas* hemocyanin (Ox-CCH) obtained by the oxidation of its carbohydrates using sodium periodate. We assessed the internalization of Ox-CCH into DCs and its immunogenicity and antitumor effects. Transmission electron microscopy showed no changes in Ox-CCH quaternary structure with respect to native CCH, although proteolytic treatment followed by SDS-PAGE analysis demonstrated that Schiff bases were formed. Interestingly, DCs internalized Ox-CCH faster than CCH, mainly through macropinocytosis. During this process, Ox-CCH remained inside endosome-like structures for a longer period. Mouse immunization experiments demonstrated that Ox-CCH is more immunogenic and a better carrier than CCH. Moreover, Ox-CCH showed a significant antitumor effect in the B16F10 melanoma model similar to that produced by CCH, inducing IFN-γ secretion. Together, these data demonstrate that the aldehydes formed by the periodate oxidation of sugar moieties stabilizes the CCH structure, increasing its adjuvant/immunostimulatory carrier effects.**

**Keywords:** Antitumor effect • *Concholepas* hemocyanin • DCs • Immunogenicity • Periodate oxidation

## Introduction

Hemocyanins are large glycoproteins present in the blood of some mollusks and arthropods, and their main function is to transport oxygen to the tissues. This process is accomplished by the presence of two copper atoms coordinated to a triad of histidines in every functional unit (FU) of the molecule. Hemocyanins are large structures, with molecular weights between 4 and 8 MDa. Their basic structure is composed of ten subunits that are self-assembled into a hollow cylinder known as a decamer. In gastropods, decamers can self-associate face-to-face to form stable dimers or didecamers [1, 2]. Inoculating mammals with hemocyanins induces a strong immune response that is characterized by a Th1 cytokine profile. Because of this property, hemocyanins are commonly used as carrier proteins to promote the production of Abs against hapten molecules and peptides, as a carrier–adjuvant for therapeutic cancer vaccines, and as a nonspecific immunostimulant during superficial bladder cancer (SBC) therapy [3, 4]. Keyhole limpet hemocyanin (KLH) from the gastropod *Megathura crenulata* is the most frequently used hemocyanin for this purpose.

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The versatile properties of KLH in biomedical applications have led to increasing commercial demand and growing interest in obtaining new hemocyanins with better immunogenicities. Several hemocyanins from other species of mollusks have been studied, including *Haliotis tuberculata* [5], *Rapana thomasiana* [6], and *Concholepas concholepas* hemocyanin (CCH) [7]. Only CCH has been preclinically evaluated in a murine experimental model of SBC and may be considered as an alternative therapy for SBC [8, 9]. All these proteins, including KLH, can be obtained only from their natural source because it has not been possible to express these heterologous proteins, mainly due to their complex structure [5, 10, 11].

Although CCH and KLH have been widely used, the relationship between their structural features and their immunologic mechanisms by which they exerts their potent adjuvant/immunostimulatory effects is unclear. Despite KLH and CCH have different sources and quaternary structures, both proteins display similar immunogenicity and antitumor capability, which suggest that a conserved pattern induces an ancient immunogenic and immunostimulatory mechanism [7, 8]. Many authors have proposed different factors to explain these properties, such as the large size, the complex quaternary structure with D5-like symmetry, the xenogenicity, and the sugar moiety content of hemocyanins. However, our studies evaluating the immunosti-mulatory properties of the isolated subunits of CCH have shown that the large size and the complex quaternary structure do not determine the immunogenicity and antitumor effects of hemo-cyanins in a mouse model of SBC [12]. This observation has been supported by another report that assessed the immunogenicity of subunits isolated from *R. thomasiana* hemocyanin [13].

Specialized APCs, such as DCs, are key components of the immune system. DCs internalize, process, and present antigens to T lymphocytes via MHC class I or II. In this context, it has been demonstrated that the conformational and structural stability of a foreign protein play a crucial role on antigen proteolysis, antigen processing in APCs, T-cell stimulation and on its immunogenicity [14, 15]. The generation of immunogenic peptides is in direct relationship between the type of APC and the proteolytic enzymes involved in their formation [15, 16]. In fact, the less digestible forms of identical antigens are more immunogenic, inducing better T- and B-cell responses [17]. Some authors have reported that the introduction of reactive aldehydes groups either directly or indirectly by cross-linking reagents induces the stabilization of the structure of diverse antigens [17–19]. Among these agents, sodium periodate indirectly introduces aldehydes through the oxidation of the sugar moieties present in the antigen. This reaction has been extensively used to attach molecules to carrier proteins, to degly-cosylate glycoproteins, and also to stabilize protein structures [19–22]. In addition, it has been demonstrated that the introduction of aldehydes into OVA by the oxidation of their oligo-saccharides with sodium periodate enhances their immunogenicity [23].

Gastropod hemocyanins have a carbohydrate content of 2–9% w/w, with mannose being the major monosaccharide found in these structures [24–27]. To stabilize CCH structure, we oxidize its sugar moieties with sodium periodate in order to induce internal cross-linking within the protein. Here, we investigate the influence of an increased stabilization of CCH during its internalization and processing by DCs in vitro, and its immuno-genicity and nonspecific antitumor effect in the B16F10 mouse melanoma model.

## Results

### Periodate treatment induces internal cross-linking within CCH

To stabilize the structure of CCH, we oxidize its carbohydrates with sodium periodate to generate Schiff bases between the free amines and the reactive aldehydes formed by the oxidation procedure [28]. The chemical preparation of CCH (Ox-CCH, oxidized CCH) was used in all subsequent experiments. An analysis by transmission electron microscopy (TEM) of the negatively stained hemocyanin molecules treated with sodium periodate showed that its quaternary structure was not affected because Ox-CCH maintained the characteristic hollow cylindrical form of mollusk hemocyanins (Fig. 1A). SDS-PAGE analysis showed differences in the mobility pattern between the native and the periodate-treated hemocyanin; Ox-CCH did not enter the resolving portion of the gel (Fig. 1B). This effect was attributed to internal cross-linking within CCH by the periodate oxidation [29].

As proteolytic treatment of hemocyanins with trypsin is a procedure commonly used to study their structure, we used this enzyme to assess whether Schiff bases were formed in Ox-CCH. We hypothesized that trypsin would not be able to digest Ox-CCH because trypsin is highly selective for positive amino acids, such as arginines and lysines. Thus, native CCH and Ox-CCH were incubated with low concentrations of trypsin (0.2% w/w) for different periods of time. The results showed that the native protein was rapidly proteolyzed. By contrast, Ox-CCH was only partially proteolyzed and was retained in the stacking portion of the gel (Fig. 1C). To confirm this, we digested CCH and Ox-CCH with proteinase K (0.2% w/w), an enzyme that preferentially cleaves peptide bonds adjacent to the carboxyl groups of aliphatic and aromatic amino acids. The results indicate that CCH and Ox-CCH were equally degraded by proteinase K, confirming the formation of Schiff bases (Fig. 1D). To observe the intramolecular modification of Ox-CCH, we used SDS-PAGE analysis to compare the banding patterns of CCH and Ox-CCH challenged with a high concentration of trypsin (1% w/w) for a longer time (4h). The SDS-PAGE analysis showed that CCH treatment produced different digestion patterns (Fig. 1E).

Altogether, these data indicate that the periodate oxidation procedure generated local changes within CCH due to the formation of Schiff bases although its quaternary structure was not affected. Using this modified and more stable protein, we

 

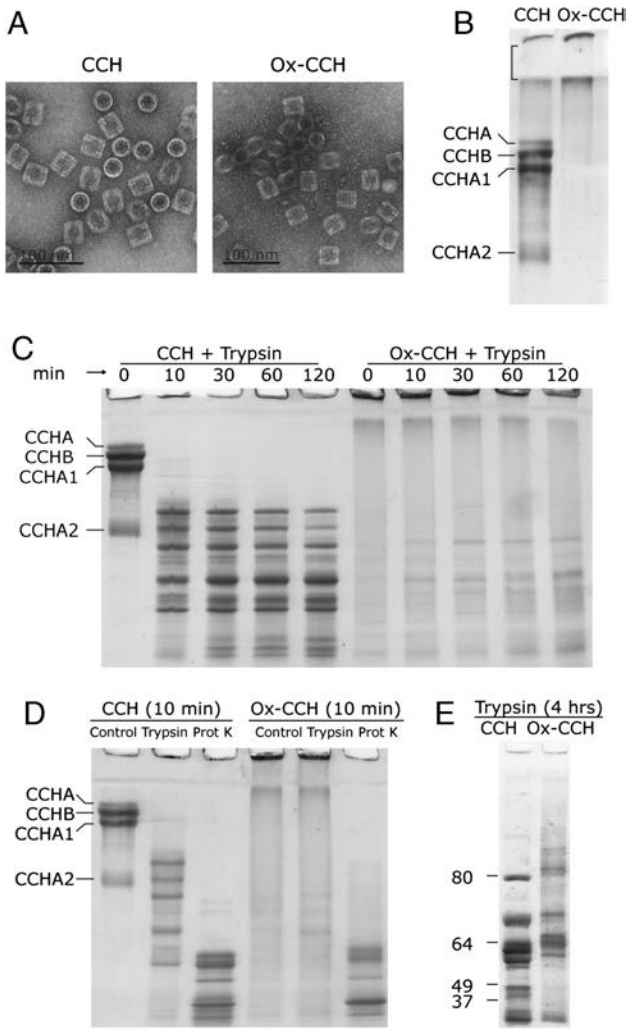


**Figure 1.** Effects of sodium metaperiodate oxidation in *Concholepas* hemocyanin (CCH). (A) Periodate treatment of CCH did not affect its quaternary structure. Negative staining of native CCH and Ox-CCH. The images show the top (circles) and lateral (rectangles) views of hemocyanin molecules observed by TEM. The side views show the characteristic didecameric form of mollusk hemocyanins, with subunits arranged in layers. Representative image are from at least three independent experiments. (B) Periodate treatment of CCH modified its electrophoretic migration patterns. CCH and Ox-CCH were analyzed by SDS-PAGE gradient gel (5–15%) and visualized with silver staining. CCH showed a characteristic banding pattern (CCHA, 405kDa; CCHB, 350kDa; CCHA1, 300kDa, and CCHA2, 108kDa). The stacking portion of the gel is denoted. Representative images are from six independent experiments. (C) Kinetics of the enzymatic degradation process. Native CCH and Ox-CCH were digested with trypsin (0.2%w/w) for different periods of time and resolved with an SDS-PAGE gradient gel (5–15%). The control line (0 min) shows the common banding pattern of CCH. Note that a significant fraction of Ox-CCH remained in the pocket of the stacking gel and did not enter the separating gel, in contrast to the native CCH. (D) Differential patterns of proteolysis were obtained with trypsin and proteinase K treatment. CCH and Ox-CCH were digested with trypsin and proteinase K (0.2%w/w) for 10 min at 37°C and resolved with a SDS-PAGE gradient gel (5–15%). The control line shows the characteristic banding pattern of CCH. (E) A high concentration of trypsin (1% w/w) was used for 4 h to observe the proteolytic banding patterns of CCH and Ox-CCH. (C–E) Gels were stained with Coomassie blue and are representative of three independent experiments.

evaluated the influence of the conformational stability of CCH on its immunological properties.

## BM-derived DCs recognize and endocytose Ox-CCH

Immature DCs have a high capacity to endocytose antigens through two different mechanisms: fluid phase macropinocytosis and clathrin-coated pits [30–32]. We used BM-derived DCs (BMDCs) to determine if the modifications of CCH have an impact in its recognition and internalization by these cells. BMDCs were exposed to CCH and Ox-CCH for 3 and 24 h and then permeabilized to analyze the internalization of these molecules by flow cytometry. After 3 h of incubation, Ox-CCH was endocytosed significantly faster than CCH and, at 24 h of treatment, both proteins were detected inside these cells with similar fluorescence intensities (Fig. 2A). These results were confirmed with TEM. Because of the large size of CCH, which is approximately 325Å in diameter and 392Å in height, and because of its peculiar structure as a hollow cylinder [7, 8], we were able to unequivocally identify the presence of hemocyanin molecules (Fig. 2B and C). According to our previous data, macropinocytosis is the main endocytosis mechanism used by BMDCs to uptake hemocyanins. This fact is made evident by the visualization of cell-surface ruffling (Fig. 2B1 and B2 for CCH; Fig. 2B4–B6 for Ox-CCH) and superficial vacuoles containing hemocyanin molecules (Fig. 2B2 and B3 for CCH; Fig. 2B4 for Ox-CCH). However, CCH and Ox-CCH were incorporated by clathrin-coated pits to a minor extent (Fig. 2C2 and C5, respectively) because these molecules were found in coated vesicles (Fig. 2C3, C4, and C6, respectively). Although hemocyanins are internalized and processed, the BMDCs did not mature in vitro (evaluated as upregulation of MHC II and costimulatory molecules by flow cytometry) after up to 72 h of incubation with CCH and Ox-CCH (data not shown). This result has also been reported in mouse DCs primed with CCH in vivo [33].

Summarizing our current results, we demonstrated that BMDCs uptake indistinctly native and periodate-treated CCH. The main process involved in hemocyanin internalization is macropinocytosis.

## Ox-CCH is more immunogenic than native CCH

As mentioned, hemocyanins are known to be excellent carrier proteins for producing Abs against peptides and haptens. In this context, we evaluated the roles of CCH and Ox-CCH in the humoral response of immunized mice without adding adjuvants. The primary humoral immune response showed that the Ab titer against Ox-CCH was stable for a longer period of time than the titer against CCH 19 days postimmunization (Fig. 3A). Moreover, the secondary humoral immune response indicated that Ox-CCH and its subunits (Ox-CCHA and Ox-CCHB) (CCHA, subunit A of CCH; CCHB, subunit B of CCH) induced a significantly greater Ab titer than did their native counterparts in C57BL/6 (Fig. 3B). We

 

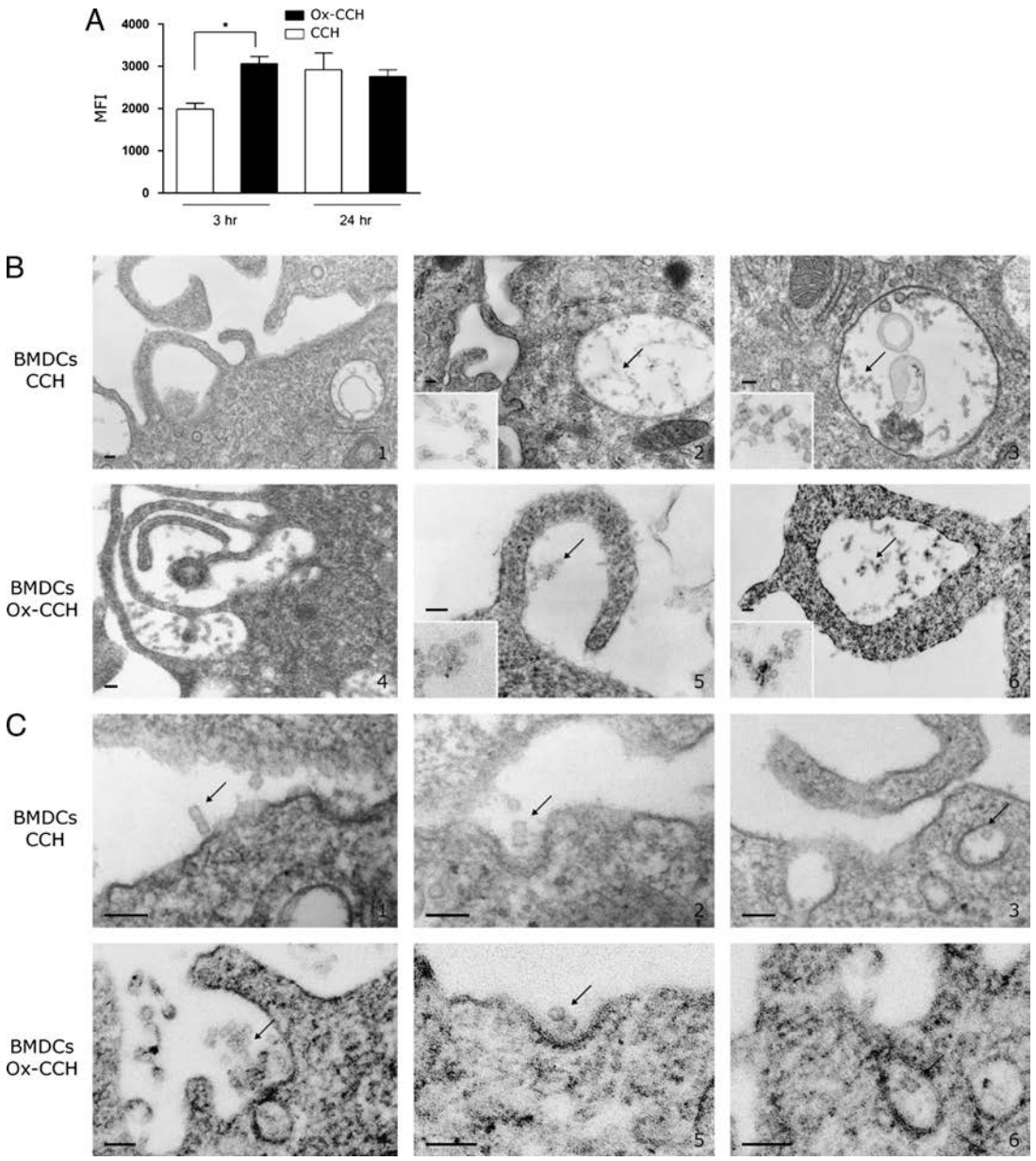


**Figure 2.** BMDCs recognize and take up Ox-CCH faster than CCH. (A) Ox-CCH was endocytosed faster than native CCH by BMDCs. Cells were treated with CCH or Ox-CCH at 37°C for 3 and 24h. Cells were then permeabilized with PBS-0.05% Triton X-100 and exposed to a rabbit antiserum raised against CCH (at a serum dilution of 1:160, which recognizes CCH and Ox-CCH with equal intensities). Cells were revealed with an anti-rabbit IgG-FITC antiserum and analyzed by flow cytometry. Data are shown as mean + SEM and are representative of four independent experiments $^{*}p < 0.05$ between CCH and Ox-CCH, one-way ANOVA, and Bonferroni post-test. (B, C) DCs internalized Ox-CCH and native CCH mainly by (B) macropinocytosis but also by (C) clathrin-mediated endocytosis. TEM analysis of BMDCs incubated for 5 min with CCH and Ox-CCH. Scale bar represents 300 nm. Hemocyanin molecules are indicated by arrows. Representative images are from two independent experiments. Higher magnification inset images of hemocyanin molecules in the area are indicated with arrows on the main images. Bar represents 100 nm.

also evaluated the behavior of Ox-CCH as a carrier protein. CCH and Ox-CCH were coupled with human IgG using a classical immunization protocol with complete and incomplete Freud's adjuvant in BALB/c mice. We observed that Ox-CCH significantly improved the humoral response against a human IgG compared with CCH (Fig. 3C). Taken together, the above data demonstrated that the conformational stabilization of CCH by the periodate treatment significantly augmented its immunogenicity.

## Periodate treatment does not affect the antitumor properties of CCH

In vivo, KLH and CCH have been demonstrated to be useful proteins for SBC therapy. However, they have been poorly explored in other types of cancers. It has been reported that KLH (200 μg/dose) inhibits the appearance of a melanoma tumor until 16 days after HTB68 cell implantation [34]. Therefore, we

 

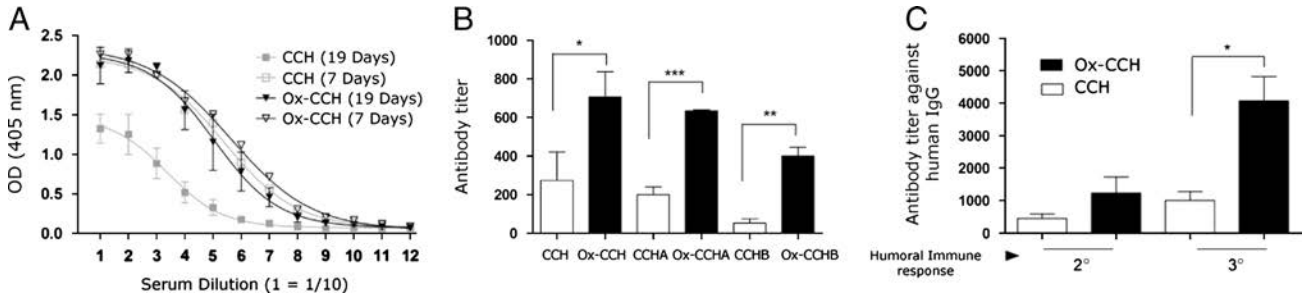


**Figure 3.** Ox-CCH is more immunogenic than its native counterpart. (A) C57BL/6 mice were primed i.p. with 200 $\mu g$ of CCH or Ox-CCH, and the primary immune response was evaluated by an indirect ELISA on days 7 and 19. Data are shown as mean + SEM of $n = 5$ mice per group and are representative of two independent experiments. (B) C57BL/6 mice were primed i.p. with 200 $\mu$g of Ox-CCH or CCH as control. Periodate-treated CCH subunits (Ox-CCHA and Ox-CCHB) were evaluated and their native counterparts used as controls (CCHA and CCHB, respectively). Fifteen days later, the mice were immunized again and then bled 10 days later to analyze the specific secondary immune response by an indirect ELISA in which the individual immunogens were used as serologic target. The Ab titer was defined as the reciprocal of the serum dilution that showed half of the maximum absorbance at 405 nm. Data are shown as mean + SEM of $n = 3$ mice per group, $^{*}p < 0.05$ between CCH and Ox-CCH, $^{***}p < 0.001$ between CCHA and Ox-CCHA, and $^{**}p < 0.01$ between CCHB and Ox-CCHB, one-way ANOVA, and Bonferroni post-test. (C) Ox-CCH is a better carrier protein than CCH. Both forms of *Concholepas* hemocyanin were coupled to human IgG. BALB/c mice were immunized with 100 $\mu g$ of each preparation in the presence of CFA (primary immunization) or incomplete Freund's adjuvant (secondary and tertiary immunizations). Anti-human IgG titers were determined by an indirect ELISA at 10 days after secondary and tertiary immunization. Data are shown as mean + SEM of one experiment with $n = 3$ mice per group. $^{*}p < 0.05$ between CCH and Ox-CCH one-way ANOVA and Bonferroni post-test.

investigated the role of the modified CCH in the antitumor effect of CCH in the B16F10 murine melanoma model. Mice previously primed with native CCH or Ox-CCH were challenged with B16F10 melanoma cells injected into the flank. The mice then underwent intralesional therapy with each hemocyanin during a period of six consecutive days (Fig. 4A) in a similar manner to those described in the previous studies of SBC [8]. The results showed that both proteins induced a significant retardation of the tumor growth, and Ox-CCH was more efficient than CCH (Fig. 4B). The survival curves showed similar behaviors for both hemocyanins. However, Ox-CCH tended to be more effective than CCH (Fig. 4C). It is important to note that all the control mice died at day 35 of the bioassay. By contrast, the CCH- and Ox-CCH-treated mice showed survival rates of 15 and 25%, respectively, on day 60. In some experiments, survival rates were followed until 3 months, with similar results (data not shown). Throughout our studies with both proteins, we did not observe allergic reactions or toxic effects in the mice.

Next, we studied the humoral immune response of mice against Ox-CCH. To achieve this objective, mice were bled on day 13 of the bioassay and the humoral immune response against hemocyanins was evaluated. The higher immunogenicity of Ox-CCH compared with that of CCH that had been seen in the previous experiments was confirmed (Fig. 4D). To determine if the increases in Ab titers against Ox-CCH were due to changes in their antigenicity, we evaluated the cross-reactivity between native and modified CCH by ELISA. We observed that anti-CCH mouse sera reacted more strongly with Ox-CCH than anti-Ox-CCH mouse sera reacted with CCH, indicating that the immunogenicity and antigenicity were improved when Ox-CCH was used as an immunogen (Fig. 4E).

In this context, it has been demonstrated that CCH induce a characteristic Th1 humoral immune response that is characterized by a significant increase in the IgG2a and IgG2b subclasses of Abs in mice receiving immunotherapy for SBC [8]. Nevertheless, mice with melanoma tumors showed high levels of IgG1 and IgG2a subclasses that Th1-(IgG2a, IgG2b, and IgG3) but also Th2 (IgG1)-cell responses were induced (Fig. 4F).

In addition, due to the capacity of hemocyanins to induce IFN-$\gamma$ and the ability of this cytokine to prevent the formation of tumors [35], we investigated the participation of the stability of Ox-CCH during the production of IFN-$\gamma$. The data showed similar levels of this cytokine when native CCH or Ox-CCH were used (Fig. 4G).

These data showed, for the first time, the ability of CCH and Ox-CCH to generate an antitumor immune responses toward a Th1-cell response in the B16F10 melanoma model, and also we demonstrate that the periodate treatment does not affect this property. It is important to note that the antitumor effect of Ox-CCH and CCH might be improved. In addition, different factors including, model optimization regarding the tumor cell number, optimal dose of both forms of hemocyanin and immunization schedule deserve our detailed evaluation in the future.

## The limited processing of Ox-CCH by DCs increases its immunogenicity

Antigen immunogenicity is closely associated with its processing by APCs. These cells catabolize the majority of antigens and present them to T lymphocytes. However, some antigens remain protected from degradation either while in intracellular compartments or bound to the cell surface [17,36]. We proposed that the high immunogenicity observed for Ox-CCH might be associated with differential processing by DCs. Thus, we analyzed the antigen-processing kinetics by BMDCs for CCH and OVA, a widely used model antigen. The immunoblot showed that after 72 h of coincubating CCH and BMDCs, a band of approximately 49 kDa was observed, which likely corresponded to one FU. This result indicated that the BMDCs could not fully process CCH. In

 

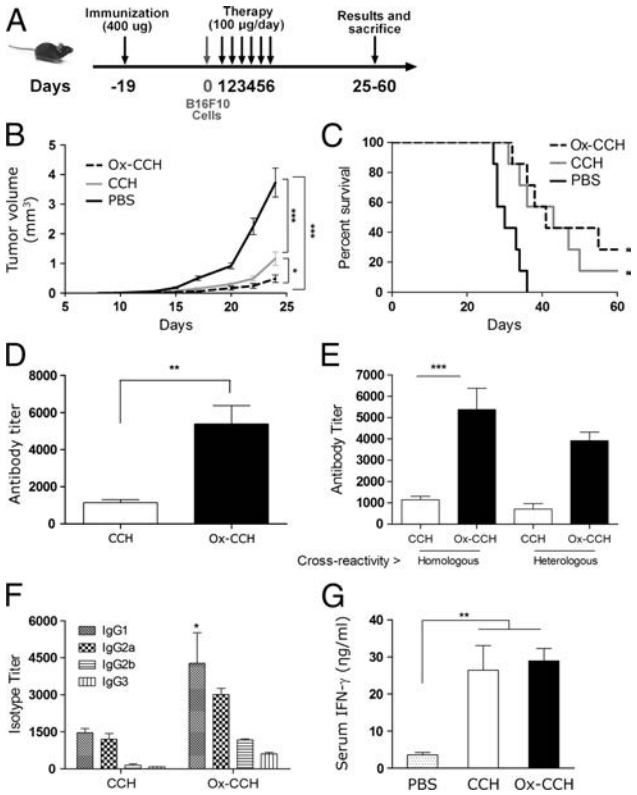


**Figure 4.** The periodate treatment did not affect the antitumor effect of CCH in the B16F10 mouse melanoma model. (A) Schedule schematic of the bioassay. Groups of eight to eleven mice were s.c. immunized with 400 µg of Ox-CCH or CCH in PBS, challenged with $1.5 \times 10^5$ B16F10 melanoma cells, and exposed to intralesional therapy with the respective proteins or PBS for six consecutive days. Survival was followed up to 60 days. (B) Effect of Ox-CCH on tumor growth. Tumor size was measured every 3 days until day 25 previous to the exponential growth of the tumor. Tumor volume was calculated using ellipsoid formula. Representative image of three independent experiments, $^*p < 0.05$ between CCH and Ox-CCH and $^{***}p < 0.001$ for both hemocyanins versus the control. (C) Effect of Ox-CCH on animal survival. $^*p < 0.05$ between hemocyanins and PBS, Kaplan-Meier analysis, and log-rank test. (D) Effect of Ox-CCH on the humoral immune response. Indirect ELISA assay was performed on the 13th day of the bioassay. $n = 3$, $^{**}p < 0.01$ between CCH and Ox-CCH, one-way ANOVA and Bonferroni post-test. (E) Effect of periodate treatment of CCH on its immunogenicity and antigenicity. ELISA plates were coated with CCH or Ox-CCH to measure the cross-reaction between these molecules. $n = 3$, $^{***}p < 0.001$ between CCH and Ox-CCH, one-way ANOVA and Bonferroni post-test. (F) Ox-CCH-specific IgG subclass production. The IgG subclasses were determined by an isotype-specific ELISA from mouse sera collected on the 13th day of the bioassay. $n = 3$, $^*p < 0.05$ between IgG1 and IgG2a, one-way ANOVA and Bonferroni post-test. (G) IFN-γ determination. The results show the levels of IFN-γ determined by ELISA, on sera of mice collected 13 days after the injection of B16F10 cells. $n = 3$, $^{**}p < 0.01$ between CCH and Ox-CCH versus the control with PBS, one-way ANOVA and Bonferroni post-test.

contrast, OVA was completely degraded (Fig. 5A). Although CCH and Ox-CCH had different rates of internalization at early time points (Fig. 2A), both showed similar processing patterns at 24 and 72h of incubation with BMDCs, as observed by western blot analysis. The 49-kDa band persisted in both samples (Fig. 5B). Unanue [36] had previously demonstrated that the immunogenicity of KLH depends, in part, on its bond to the plasma membranes of APCs. Thus, we observed that Ox-CCH was more strongly bound to the extracellular membranes of BMDCs (Fig. 5C) and accumulated as whole molecules for a longer time inside endosome-like structures compared with CCH (Fig. 5D). Taken together, these results show that CCH is processed slowly and strongly suggest that the persistence and limited processing of Ox-CCH in APCs might explain its high immunogenicity.

## Discussion

We found that hemocyanins can enhance their immunological properties by chemical modification of their structure. Our results indicate that Ox-CCH was significantly more immunogenic than CCH, both in its ability to induce the production of specific Abs and in its use as a carrier protein. These differences might be explained by the higher structural and conformational stability of Ox-CCH [37]. Intramolecular cross-linking has shown to increase thermal stability of different proteins [38,39]. However, protein stabilization by internal cross-linking agents is still controversial. It has been reported that some enzymes and toxins are inactivated by mechanisms involving either oxidation of specific amino acids and formation of disulfide bonds after periodate and formaldehyde treatments [40–42]. In addition, some authors have described that increasing protein conformational stability might either increase [17] or diminish the immunogenicity of certain antigens [43–45]. The question that arises is why Ox-CCH presents higher immunogenicity than CCH. It is possible that the major exposure of epitopes in Ox-CCH may allow them, after slow processing by APCs, to generate numerous peptides that would fit with high affinity in most MHC II haplotypes involved in the stimulation of B lymphocytes by T lymphocytes. This binding would lead to an increase in the production of Abs. In this respect, we have previously demonstrated that anti-CCH mAbs improved their binding to Ox-CCH after periodate treatment, indicating that the polypeptide epitopes of Ox-CCH are better exposed after this treatment, which increases Ox-CCH antigenicity [46].

In addition, the reactive aldehydes formed by the oxidation of CCH oligosaccharides might play a role in the immunogenicity of Ox-CCH. It has been reported that different carbohydrates oxidized with periodate and compounds with free aldehydes have antitumor effects in some types of cancers [47–49]. In this respect, a synthetic Schiff base drug known as tucaresol potentiates the immune system by enhancing $CD4^+$ lymphocyte activation, which leads to the stimulation of Th1-type cytokine production. This process is accomplished by the formation of a Schiff base between the free aldehyde of tucaresol and the amines lying on the surface of T cells, which leads to the activation of a $K^+$ channel that increases MAPK activation. This process promotes the secretion of Th1 cytokines, such as IL-2 and IFN-γ [49–51]. On the other hand, Delamarre et al. demonstrated that the fixation of proteins with aldehydes enhanced their resistance to lysosomal proteolysis and therefore increased their immunogenicity [17]. In this case, the aldehyde groups induced the protein stability by the formation of Schiff bases, which restricted the protein's

 

susceptibility to lysosomal proteolysis. We observed Ox-CCH molecules for longer periods than native CCH in endosome-like structures and on the surface of BMDCs, demonstrating the higher resistance of Ox-CCH molecules to enzymatic degradation and antigenic processing. We cannot exclude the possibility that Ox-CCH and CCH may be stored in different lysosomal compartments. Indeed, it has been recently described that some DCs acquire small particles that are stored in lysosomal compartments with a reduced capacity for degradation, resulting in prolonged antigen presentation either to T or to B cells [52].

Considering these results, we propose that the Schiff bases formed by the periodate oxidation of the carbohydrate moieties in the present study induced the stabilization of Ox-CCH, increasing its enzymatic resistance and its bounding to the extracellular membrane, thereby limiting its susceptibility to lysosomal proteolysis and favoring the presentation of antigens to B cells (as an intact molecule on the plasma membrane) and to T lymphocytes (as a processed peptide) in lymphoid organs for longer periods of time. This process would result in augmented immunogenicity.

On the other hand, periodate oxidation treatment has been used to deglycosylate glycoproteins. Hemocyanins present high percentages of mannose residues that have been reported to contribute to their internalization on APCs and directly in the maturation of DCs [24, 27, 53]. Our data suggest that hemocyanins sugar moieties are not essential for their immunogenicity and non-specific immunostimulatory effects because periodic acid Schiff (PAS) staining showed that all the oligosaccharide moieties were eliminated from Ox-CCH (data not shown). However, this procedure lacks the ability to dissect the immunologic role of the glycans on CCH.

Currently, hemocyanins are extensively used in biotechnology and biomedicine as carrier proteins to produce Abs against haptens and peptide molecules. In fact, several cutting-edge therapeutic vaccines for cancer, including melanomas, neuroblastoma, sarcomas, and cancers of the breast, prostate, ovary, and lung tissues, utilize hemocyanins as carrier–adjuvants to generate Abs against different tumor-associated antigens (TAAs). Such TAAs include gangliosides and mucin-like epitopes, which are used to eliminate cancer recurrence by circulating tumor cells and micrometastases [54–56]. Surprisingly, our results demonstrate that periodate treatment of CCH enhanced its carrier protein capacity. Therefore, we hypothesize that the high immunogenicity induced

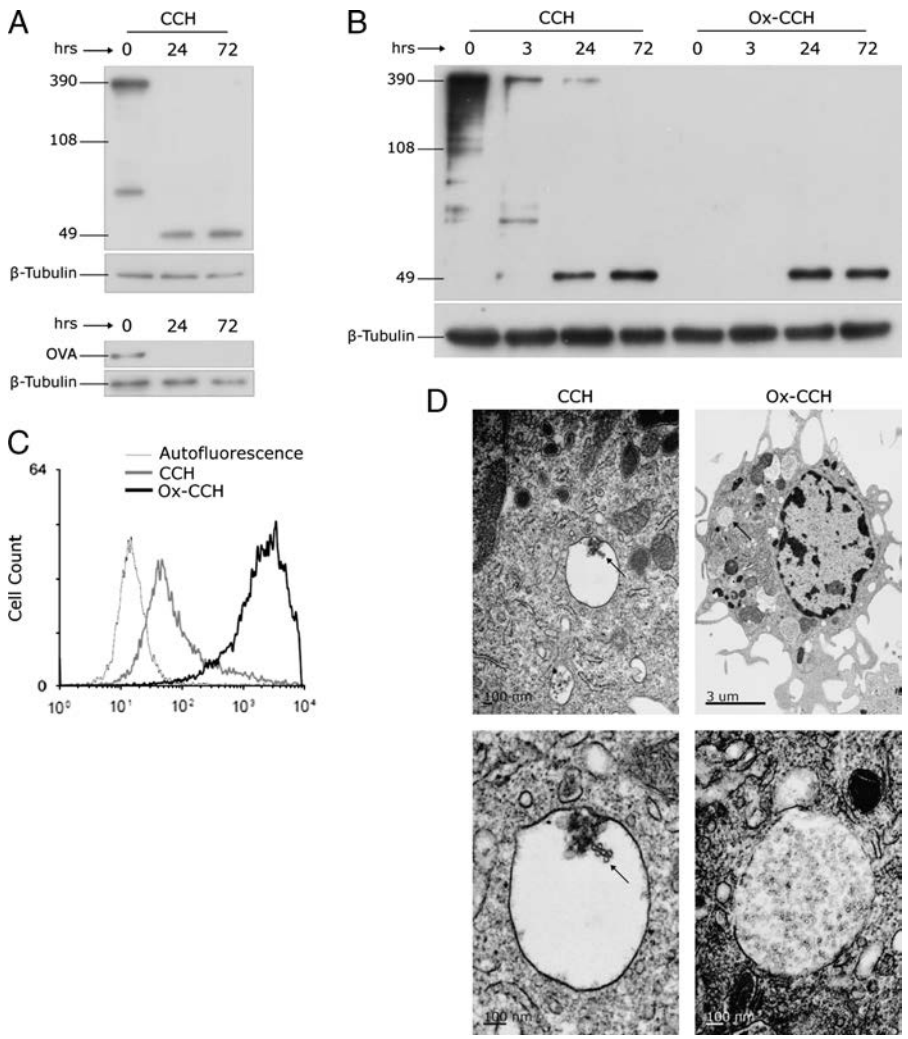


**Figure 5.** Ox-CCH is slowly processed and accumulated in the plasmatic membrane and endosome-like structures of BMDCs cultured in vitro. (A) Analysis of the processing patterns of CCH and OVA. BMDCs were exposed to 100 μg/mL of CCH or OVA for the indicated times, and the cell extracts were immunoblotted with an anti-CCH Mab or a mixture of anti-OVA mAbs, followed by chemiluminescent detection. An anti-β-tubulin Ab was used as a loading control. The results are representative of three independent experiments. (B) Analysis of the processing patterns of CCH and Ox-CCH. DCs were exposed to 100 μg/mL of CCH or Ox-CCH for the indicated times. The cell extracts were immunoblotted with an anti-CCH mAb, followed by chemiluminescent detection. The results are representative of three independent experiments. Anti-β-tubulin Ab was used as a loading control. (C, D) Ox-CCH was slowly processed and accumulated in the extracellular membrane and endosome-like structures, respectively. BMDCs were incubated for 24 h with CCH or Ox-CCH and analyzed by (C) flow cytometry (n = 4) and (D) TEM (n = 3). Lower and higher magnification micrographs show hemocyanin molecules (arrows) in endosome-like structures.

 

by Ox-CCH might be explored more profoundly as a TAAs carrier at the preclinical level. This is a significant issue because one of the main problems with these vaccines is the consistent induction of high titers of IgM and IgG Ab responses against TAAs [3, 4].

Finally, we conclude that the amino acid sequences and the conformational stability of hemocyanins may give clues to their adjuvant/immunostimulatory properties. Despite the differences between CCH and KLH, both proteins may have similar amino acid motifs and domains that drive and stimulate the immune responses toward a strong Th1 profile. This idea is based on the presence of regions of high-sequence homology in gastropod hemocyanins, which are especially clear in the active site region [1, 57], and are confirmed by the presence of common or mimic epitopes that were revealed by Ab cross-reactivity among CCH and KLH [46]. Our future studies will focus on the discovery of the common immunodominant motifs of hemocyanins that are responsible for their immunomodulatory effects in mammals.

## Materials and methods

### Hemocyanins

Hemocyanin from *C. concholepas* (Immunocyanin) that was isolated under sterile and pyrogen-free conditions was provided by Biosonda (Santiago, Chile). CCH subunits (CCHA and CCHB) were purified according to the methods described by Becker et al. [12]. All solutions containing hemocyanins and buffers for their dialysis were maintained at 4°C, prepared with water for irrigation (Baxter Healthcare, Charlotte, NC, USA) and filtered through a 0.02-μm membrane filter (Millipore, Billerica, MA, USA).

### Mice

C57BL/6 and BALB/c mice were obtained from the Universidad de Chile and BiosChile Ingeniería Genética SA, respectively. Experimental mice (8- to 12-wk old) were housed at 22–24°C with a light/dark cycle of 12/12 h. The experiments were performed in compliance with institutional guidelines for the welfare and treatment of animals.

### Cell lines

Mouse B16F10 melanoma cells were donated by Dr. Flavio Salazar-Onfray (Universidad de Chile). The cells were grown at 37°C in a humidified atmosphere (10% $CO_2$ in air) in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; HyClone, St. Louis, MO, USA), 100U/mL penicillin, 100μg/mL streptomycin, 2.5μg/mL Fungizone, 1mM sodium pyruvate, and 0.1 mM nonessential amino acids (Invitrogen).

### Preparation of BMDCs

BMDCs from female C57BL/6 mice were prepared according to the method of Inaba et al. [58] with modifications. Briefly, BM was flushed from tibias and femurs and depleted of red blood cells using ammonium chloride. Then, BM cells were seeded at a density of $1 \times 10^6$cells/mL in 24-well plates and cultured for 6 days at 37°C in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100U/mL *penicillin,* 100μg/mL *streptomycin,* 50μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 1mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10ng/mL granulocyte-macrophage CSF (GM-CSF) (BD Diagnostics, Franklin Lakes, NJ, USA). The culture medium was replaced with fresh medium after 48 and 96 h of culture. BMDCs were purified after 6 days of culture by MACS-positive selection with CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Then, the isolated DCs were cultured at $1 \times 10^6$cells/mL in a 24-well plate in the same culture medium.

### Chemical modification of CCH

The chemical oxidation with sodium periodate method was utilized to modify CCH and the CCHA and CCHB subunits [59]. Briefly, each protein (1–2mg/mL) was dissolved in 0.1 M sodium acetate buffer (pH = 5.5) containing 15 mM sodium periodate (Merck, Darmstadt, Germany) and incubated for 1 h in the dark at room temperature. Next, 25 μL of a solution of ethylene glycol (Merck) was added to each 2mL of protein and incubated overnight at 4°C. Finally, the preparations were concentrated using an Amicon Ultra-15 and an Amicon Ultra-4 (Millipore), dialyzed against PBS at 4°C, and filtered with a 0.22-μm membrane filter.

### Protease digestion of hemocyanins

Modified CCH and its respective native forms (as controls) were digested with trypsin and proteinase K (Sigma-Aldrich) at a concentration of 1 or 0.2%w/w, respectively. The enzymatic reaction was performed at 37°C for various times in a buffer containing 50 mM Tris (pH = 8.8) and stopped with a 1% phenylmethylsulfonyl fluoride solution [60] (PMSF, Merck).

### SDS-PAGE and western blotting

Hemocyanin samples were mixed with a buffer containing SDS and 4% β-mercaptoethanol, heated for 5 min at 100°C, and analyzed by SDS-PAGE on a gradient (5–15%) or 10% polyacrylamide separating gel, as described by Laemmli [61]. The gels were developed with either Coomassie blue or silver staining, as described previously for KLH [7, 8]). For Western blots, polyacrylamide gels with hemocyanin samples were transferred to a

 

0.45-μm pore nitrocellulose membrane (Pierce-Endogen) at 300 mA for 1.5 h. The membranes were incubated overnight at 4°C with 1% PBS-bovine serum albumin (BSA) and then incubated for 2h with hybridoma supernatants containing the anti-CCH 2H12 monoclonal Ab (mAb) [46]. After washing with 0.02% PBS-Tween, the membranes were incubated for 1 h at room temperature with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, Pierce-Endogen). Finally, the membranes were developed with a chemiluminescent substrate (West Pico Chemiluminescent Substrate, Pierce-Endogen).

### Transmission electron microscopy

Native and periodate-treated hemocyanin samples were negatively stained according to the method described by De Ioannes et al. [7, 8]. Briefly, aliquots of protein samples (100 μg/mL) were applied to parlodion-coated copper grids that had been previously stabilized by vacuum evaporation on a carbon coat. The proteins were stained with 1–2% aqueous uranyl acetate solution. To study hemocyanin endocytosis, $5 \times 10^5$ BMDCs that had been previously treated with hemocyanins were centrifuged at 2000 rpm for 3 min, washed with PBS, and fixed in a solution of 2% glutaraldehyde (Polysciences, Warrington, PA, USA) in 0.1-M sodium cacodylate solution (pH = 7.4). The cells were postfixed with $OsO_4$, dehydrated, and embedded in Epon (Polyscience) according to the method of Luft [62]. Then, the samples were stained with lead citrate according to the procedure of Reynolds [63]. The preparations were examined and photographed at 80 kV with a Philips Tecnai 12 electron microscope (Electronic Microscopy Facility, Pontificia Universidad Católica de Chile).

### Hemocyanin endocytosis assays

To measure the internalization rates of CCH and Ox-CCH, BMDCs were incubated with 100 μg/mL of each protein at 37°C for 3 or 24 h. Hemocyanin uptake was stopped by washing with PBS. Then, the cells were harvested and fixed as described above. The BMDCs were then permeabilized with PBS − 0.05% Triton X-100 for 20 min at room temperature and incubated with polyclonal rabbit anti-CCH serum (diluted, 1:160) for 30 min at 4°C. At this dilution, the Abs in the serum recognized CCH and Ox-CCH with the same intensity, as determined by an indirect ELISA. Then, the cells were centrifuged, washed with PBS containing 2% FBS, and incubated with an anti-rabbit IgG-FITC antiserum. All samples were acquired on a FACScan flow cytometer (Becton Dickinson, USA) and analyzed using WinMDI software.

To determine the processing pattern of CCH, Ox-CCH, and OVA (Sigma-Aldrich), BMDCs were incubated with 100 μg/mL of each protein at 37°C for 3, 24, and 72 h. For the electrophoretic analysis of BMDC extracts, the cells were washed with PBS, harvested with trypsin/EDTA, and lysed with a low detergent buffer (20 mM Tris/HCl pH 7.5; 2 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100). DC extracts were immunoblotted and visualized with an anti-CCH 2H12 Mab [46], as described above. A mixture of four mAbs developed in our laboratory (anti-OVA 2A7, anti-OVA 3G11, anti-OVA 3E4, and anti-OVA 7C11) was used with samples containing OVA. An anti-β-tubulin Ab (Sigma-Aldrich) was used as a loading control.

### Immunization schedule and humoral immune response assessment of Ox-CCH

To determine the effect of the structure stabilization of CCH on specific Ab production, a group of three 2-month-old C57BL/6 mice were immunized as follows: on day 1, they received 200 μg of Ox-CCHA or CCH in 100 μL of PBS-i.p. On day 15, the same immunization was performed. Ten days after the second or third antigen injection, the mice were bled and sera were obtained. The presence of specific IgG Abs in mouse serum was detected by an indirect ELISA, as described below. To prepare hemocyanin conjugates, Ox-CCH (10 mg) or CCH was incubated with 10 mg of human IgG proteins (Grifols, Los Angeles, CA, USA) in 3mL of 100mM borate buffer (pH = 10). Then, a 0.3% glutaraldehyde solution was slowly added to the original solution, and the mixture was left in the dark for 2 h at room temperature. The reaction was stopped in the dark for 30 min with 250 μL of 1 M glycine. The samples were dialyzed against sterile PBS at 4°C [64]. BALB/c mice were injected i.p. with 100 μg of the carrier protein in the presence of complete Freund's adjuvant (CFA, Pierce-Endogen) for the first immunization (day 1) and with incomplete Freund's adjuvant (IFA, Pierce-Endogen) for the second (day 18) and third (day 53) immunizations.

### Ab isotype determination

Anti-hemocyanin and anti-isotype Abs were determined by the procedure of Moltedo et al. [8]. ELISA plate was activated overnight with CCH and Ox-CCH (10 μg/mL). The plate was blocked for 1 h at 37°C with PBS containing 1% BSA and incubated with serial dilutions of the serum from sensitized mice or bioassay mice. Subsequently, the plate was washed with PBS containing 0.02% Tween-20 and incubated with an anti-IgG Ab coupled to alkaline phosphatase (ALP, Pierce-Endogen). In the case of the isotypes, anti-IgG1-ALP, -IgG2a-ALP, -IgG2b-ALP, and -IgG3-ALP Abs were used (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Immunotherapy experiments to assess antitumor activity

To measure the antitumor effects of CCH and Ox-CCH, the general procedure of Moltedo et al. was used [8]. Briefly, groups of C57Bl/6 mice (2–4 months) were primed with an s.c. injection of 400 μg of Ox-CCH or CCH (or 100 μL PBS) in the right flank. Twenty days later, the mice were challenged in the same region

 

with an s.c. injection of 1.5–2 $\times$ $10^5$ B16F10 melanoma cells. The next day, an immunization schedule was begun for six consecutive days, with Ox-CCH, CCH (100 $\mu$g), or PBS as a control. The tumor incidence was evaluated by visual inspection and palpation. Tumor dimensions (length and width) were measured every 3–5 days, up to day 25, prior to the exponential growth of the tumor. The tumor volume was calculated according to the formula 0.52 $\times$ (length $\times$ width$^2$). The survival of the mice was measured over a period of 60 days.

### IFN-$\gamma$ determination

To measure the production of serum IFN-$\gamma$, mice were bled at day 13 of the antitumor bioassays. The levels of IFN-$\gamma$ were determined using a commercial ELISA kit according to the manufacturer's instructions (Pierce-Endogen). Samples were measured in duplicate.

### Statistical analysis

The results of the experiments were expressed as means $\pm$ SE. Comparisons between groups were made using one-way ANOVA, and Bonferroni post-test. The survival rate was estimated by the Kaplan-Meier method and the log-rank test. Statistical significance was defined at a $p$-value of $< 0.05$. Analyses were performed using GraphPad Prism software (La Jolla, CA, USA).

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*Abbreviations:* **BMDC**: BM-derived DC · **CCH**: *Concholepas concholepas hemocyanin* · **CCHA**: subunit A of CCH · **CCHB**: subunit B of CCH · **FU**: functional unit · **Ox-CCH**: oxidized CCH · **KLH**: keyhole limpet hemocyanin · **SBC**: superficial bladder cancer · **TAA**: tumor-associated antigen · **TEM**: transmission electron microscopy

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