Functional role of CD40 and CD154 costimulatory signals in IgZ-mediated immunity against bacterial infection

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ABSTRACT

CD40 and CD154 are one of the best-characterized costimulatory molecules essential for adaptive immunity, which extensively involved in T and B cell activation, IgM Ab production, isotype class switching, germinal center formation and affinity maturation. However, the functionality of CD40 and CD154 in IgZ-mediated immunity remains limited. In this study, we explored the regulatory role of Cd40-Cd154 interaction in IgZ-mediated antibacterial immunity in zebrafish. The results showed that the IgZ-mediated antibacterial response can be significantly induced in response to A. hydrophila infection. The percentage of Cd40+ IgZ B cells and the production of IgZ Ab were substantially increased upon A. hydrophila stimulation, but these reactions were markedly declined in Cd40+ blockade fish by administering anti-Cd40 Ab or recombinant scCd40-Ig protein, accompanied with the impairment of the vaccine-initiated IgZ-mediated immunoprotection of fish against A. hydrophila infection. These observations suggested the essential role of Cd40-Cd154 interaction in IgZ-mediated bacterial immunity. Notably, the Cd40 and Cd154 costimulatory signals are required for a TD antigen-induced IgZ immunity, but are not indispensable for a TI antigen-induced IgZ immune response. These findings indicated the differential role of Cd40-Cd154 interaction in bacterial TD and TI antigen-induced IgZ immunity, which suggested the existence of diverse regulatory mechanisms underlying IgZ-mediated antibacterial immune reactions. To our knowledge, this is the first report to show the functional role of Cd40-Cd154 costimulatory signaling pathway in IgZ-mediated immune defense against bacterial infection. We hope this study will improve the current understanding of the coevolution between the IgZ/IgT immunoglobins and Cd40/Cd154 costimulatory molecules.

1. Introduction

CD40 and CD154 are one of the best-characterized costimulatory molecules that are required for establishing adaptive humoral and cellular immunities in humans and other mammalian species [1–5]. CD40 is a 48 kDa membrane glycoprotein that is a member of the tumor necrosis factor receptor (TNFR) superfamily [6, 7]. It is expressed on many types of cells, including B lymphocytes, dendritic cells (DCs), monocytes, epithelial cells, and even endothelial cells [8, 9]. CD40 is best known as a growth signal receptor for B lymphocytes [10]. On the other hand, CD154, a ligand of CD40, is a 39 kDa membrane glycoprotein that is a member of the tumor necrosis factor (TNF) family [6, 8]. It is expressed on activated CD4+ T cells, B cells, NK (nature kill) cells, platelets and a variety of DCs [8, 11]. It is well known that interaction of CD40+ B cells with CD154+ T cells induces B cell proliferation, immunoglobulin (Ig) production, isotype class switching, germinal center formation and affinity maturation [12, 13]. In addition, CD154 has potent regulatory activities, including upregulation of costimulatory molecules (such as CD80 and CD86) on antigen-presenting cells (APCs) to help T cell proliferation and activation, and cytokine expression, which providing T cell help to B cell reactions [14–17]. In humans and mouse models, CD154-impaired patients and CD154-knockout (KO) mice may develop a severe form of immunodeficiency, named as hyper IgM syndrome, which is characterized by high level of IgM and low levels of IgA, IgG, and IgE [18]. Thus, the Ig deficiencies in CD154-deficient organisms served to underscore the central role of CD40 in mature B cell functions [19]. Besides, the CD40-CD154 interaction was also found to play important roles in the regulation of

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dendritic cell (DC)–T cell and DC–B cell crosstalk, and activation of other innate immune cells, such as macrophages and monocytes [6, 20-22]. With the development of DC vaccines, understanding the regulatory role of CD40/CD154 in DC activity will benefit to the application of DC-based immunotherapy for human diseases [23–25].

Although numerous investigations in humans and other mammalian species, the functional role of CD40 and CD154 in non-mammalian species, including teleost fish, remains poorly understood [26]. In a species, including teleost fish, the functional role of CD40 and CD154 in non-mammalian species, the functional role of CD40 and CD154 in other immunomodulatory activates in teleost fish, which will be beneficial for comprehensively understanding of the evolutionary history of these two costimulatory molecules in the early vertebrates. In the present study, we explored the costimulatory regulation of CD40-Cd154 interaction in IgZ-mediated antibacterial immunity in an Aeromonas hydrophila (A. hydrophila)-infected zebrafish model. The results showed that the Cd40-Cd154 pair are involved in IgZ immune defense against A. hydrophila infection. The IgZ antibacterial response can be induced in a thymus-dependent (TD) and a thymus-independent (TI) manner, respectively. The Cd40-Cd154 interaction was essential for a TD antigen-induced IgZ immune response, but was not required for a TI antigen-induced IgZ immunity. These observations indicated a differential role of Cd40-Cd154 signaling pathway in TD and TI antigen-induced IgZ immunity against bacterial infection, suggesting the existence of diverse regulatory mechanisms underlying IgZ-mediated antibacterial immune reactions. To our knowledge, this is the first report showing the functional role of Cd40-Cd154 costimulatory pathway in IgZ-mediated antibacterial immunity. This finding improved the current knowledge of the coevolution of IgZ immunoglobin (Ig) and Cd40/Cd154 costimulators during the evolution of adaptive immunity.

2. Materials and methods

2.1. Experimental fish and bacterial strain

Wild-type zebrafish with both sexes, 0.5–1 year old, weighing 0.5–1.0 g, body lengths 1–2 cm, were kept in recirculating water at 28 °C. All fish were kept in the laboratory for at least 2 weeks before use for the study. Only healthy zebrafish, as determined by healthy appearance and activity, were used for studies. All animal experiments were conducted in accordance with legal regulations and upon ethical approval. Aeromonas hydrophila (A. hydrophila) JBN2301 strain was kindly provided by Prof. Yang as previously described [28].

2.2. Preparation of recombinant plasmids and proteins

The extracellular domain of Cd40 and the Fc (fragment crystallizable) portion of human IgG1 were initially fused by using overselected extension PCR technique, then ligated into PET28a to construct prokaryotic expression vectors with His-tag. The primers used in this study were listed in Table S1. The PET28a-sCd154 and pCMVTag2B-Cd154 plasmids for recombinant sCd154 protein expression were constructed in our lab previously [26]. The pCMVTag2B-Cd154 plasmid was transfected into HEK293T cells by using polyethyleneimine (PEI). At 6~7 h post-transfection, the supernatant was discarded, and the cells were washed with serum-free buffer, then treated by 450 μl cell lysis buffer for Western and IP with protease inhibitor (P0013; Beyotime) for 30 min at 4 °C. The cells lysis products were then centrifuged for 10 min at 12,000 g, and the supernatant was collected for Western blot analysis. The pET28a-sCd40-Ig, pET28a-hlgG1Fc and pET28a-sCd154 plasmids were transformed into BL21 (DE3; TransGen Biotech) competent cells and cultured in LB medium containing kanamycin (50 mg/l; Sangon Biotech) at 37 °C with 250 rpm shaking until the OD600 reached 0.3–0.4. Then, isopropyl-β-D-thiogalactoside (IPTG, 0.1 mM; Sangon Biotech) was added to induce the production of target proteins for other 6–7 h. After ultrasonication, the recombinant proteins expressed in inclusion body were renatured and purified by nickel–nitrilotriacetic acid agarose affinity chromatography (Qiagen) in accordance with the manufacturer’s manual and detected via 12% SDS-PAGE. The A. hydrophila TcpAh protein was expressed in E. coli cells by transforming with the pET28a-tcpAh vector as previously described [28].

2.3. Preparation of polyclonal Abs

The recombinant Cd154 protein was used to prepare mouse anti-Cd154 Ab. For this procedure, the four-week-old Balb/c male mice (~25 g) were immunized with Cd154 protein (20 μg) each time in CFA (complete Freund’s adjuvant; Sigma-Aldrich) initially and then in IFA (incomplete Freund’s adjuvant; Sigma-Aldrich) for four times thereafter at biweekly intervals, as previously described [29]. Seven days after the final immunization, serum samples were collected. Ab were affinity purified by Protein A agarose Columns (Thermo Fisher Scientific), and the titer was examined by ELISA. The efficiency and specificity of the Ab were determined by 12% SDS-PAGE and Western blot analysis. Other Abs, including rabbit anti-Cd154, rabbit anti-Cd40, mouse anti-IgZ and rabbit anti-Cd4 used in this study, were prepared in our lab previously [29].

2.4. Functional evaluation of Cd40 and Cd154

For functional evaluation of Cd40/Cd154 in IgZ-mediated immunity against bacterial infection, in vivo Cd154-depletion and blockade assays were performed to obtain evidences. Zebrafish were intraperitoneal (i.p) injected with anti-Cd154 Ab (5 μg/fish), nonrelated mouse IgG (5 μg/fish) or sCD40-Ig (10 μg/fish), nonrelated hlgG1Fc (10 μg/fish) every 24 h for three times, followed by i.p. injected with formalin-inactivated A. hydrophila (1 × 10^8 CFU/fish) [26, 29]. Zebrafish that were i.p. injected with formalin-inactivated A. hydrophila (1 × 10^8 CFU/fish) were served as control. The transcriptional expressions of cd40, cd154 and igz and the proliferation of Cd40-IgZ B cells were examined on 3 and 7 days post-infection (dpi) by real-time RT-PCR and flow cytometry (FCM) analysis. The titers of antigen-specific IgZ and IgM Abs in serum or mucus of fish were detected at 7 and 21 dpi by ELISA, and the mucus were collected as previously described [30, 31]. Briefly, the zebrafish were anesthetized with MS-222 and the gill, skin, gut were obtained from each fish. The tissues were rinsed with PBS three times to remove the remaining blood and excised into pieces. Thereafter, the tissues were incubated for 12 h at 4 °C, with occasional shaking in protease inhibitor buffer (0.9 × PBS, containing 1 × protease inhibitor cocktail (Roche), pH 7.2) at a ratio of 500 mg of tissue per ml of buffer. Then the tissues were transferred into an Eppendorf tube, vigorously vortexed, centrifuged at 400 g for 10 min at 4 °C to remove cells. For functional evaluation of Cd40/Cd154 in TD/TI antigen-induced IgZ immune responses, the Cd154-depletion and blockade assays were performed as described above, followed by stimulating the fish with the protein antigen TcpAh (10 μg/fish) or lipopolysaccharide (LPS; E. coli serotypes O55:B5; Sigma-Aldrich; 10 μg/fish). To confirm that the TcpAh-induced T cell proliferation and IgZ Ab production were in a thymus-dependent manner, zebrafish were i.p. injected with cyclosporin A (CsA, 5 μg/fish, Novartis) together with the immunization of TcpAh protein. The proliferation of T cells was assessed by the percentage of Cd40+CD154+ cells via FCM analysis at 3 days post-immunization, and the titers of TcpAh-specific IgZ and IgM Abs were detected by ELISA at 7 or 21 days post-immunization.
2.5. Real-time PCR

The total RNA was extracted from gill, skin, gut, spleen and head kidney tissues by using an RNAisoPlus kit (Takara Bio) and reverse-transcribed into cDNAs by primescript RT reagent kit (perfect real time; RR037A; TAKARA). Real-time PCR was performed using a SYBR Premix Ex Taq kit (Takara Bio). The primers used in the study were shown in Table S1. The PCR program was as follows: 94 °C for 2 min; 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C to 65 °C for 20 s, and extension at 72 °C for 20 s, melting curve analysis at 95 °C for 15 s, 60 °C for 15 s, 60 °C up to 95 °C for 20 min, and 95 °C for 15 s, and cooling at 40 °C for 30 s. The relative expression levels of cd40, cd154 and igc genes were calculated using the 2−∆∆ct method with β-actin as reference gene. Each experiment was repeated at least thrice.

2.6. Cell preparation and sorting

The leukocytes were prepared from peripheral blood, spleen, and head kidney, gill, skin and gut tissues of zebrafish with or without antigen stimulation. For this procedure, the tissues were collected, cut into small pieces and pieces with cold PBS. Single-cell suspensions were obtained by gently teasing the tissues through a 200-μm nylon mesh filter. The leukocytes were enriched from the cell suspensions by centrifugation at 1,200 g for 25 min in Ficoll-Hypaque (diluted in PBS, pH 7.2), and collected from the interface layer (white layer) as previously described [29]. The activity of the isolated leukocytes was detected by trypan blue (0.4%; Sigma-Aldrich) staining, and preparations with living cells above 98% were used in the subsequent study [32]. Then the leukocytes were washed with ice-cold Ca2+/Mg2+-free HBSS twice by centrifugation at 350 g for 10 min, blocked with mouse anti-IgZ Ab (1:500) in 5% normal goat serum for 2 h at 4 °C. After incubation, the cells were gently washed thrice with D-Hank’s buffer, incubated with anti-mouse IgG magnetic beads (Thermo Scientific) for 15 min at 4 °C, and then applied to LS separation column according to the manufacturer’s instructions to separate the target cells. The positive cells were collected and blocked with 5% goat serum –γ-globulin –γ-globulin fractions with living cells above 98% were used in the subsequent study [29, 33].

2.7. Immunofluorescence staining

The total leukocytes were fixed with 2% paraformaldehyde at 25 °C for 10 min, blocked with 2% BSA, and then incubated with primary mouse anti-IgZ Ab (1:250) and rabbit anti-Cd40 Ab (1:250) at 4 °C for at least 2 h. Washing with 0.1× PBS, the cells were incubated with secondary PE-conjugated anti-mouse Ab and FITC-conjugated anti-rabbit Ab (Thermo Scientific). After washing, the cells were incubated with 100 μg/ml DAPI (Invitrogen) at room temperature and kept in darkness for 5 min to stain the nucleus. Non-specific rabbit or mouse IgG (1:250) was served as the negative control. Imaging was obtained by a two-photon laser-scanning microscope (Zeiss LSM710, Germany) with 630× magnification.

2.8. Flow cytometry analysis

The total leukocytes were collected and blocked with 5% goat serum for 2 h at 4 °C and then incubated with primary rabbit anti-Cd40 Ab (1:200) and mouse anti-IgZ Ab (1:200) or rabbit anti-Cd4 Ab (1:200) and mouse anti-Cd154 Ab (1:200) at 4 °C for 3 h. Normal rabbit/mouse IgG isotype was used as negative control. Thereafter, the cells were washed thrice with D-Hank’s buffer, incubated with secondary Ab (PE conjugated goat anti-mouse and FITC conjugated goat anti-rabbit, Thermo Scientific) for 1 h at 4 °C. At last, the cells were examined using a FACScan flow cytometer (BD Biosciences). At least 10,000 cells were collected from the lymphocyte gate for analysis. The gating strategy for lymphocyte lineage in leukocytes was based on the forward scatter (FSC) and side scatter (SSC) characteristics as previously described [34, 35]. Cell Quest software (BD Biosciences) and Flowjo v10.0 were used for analysis.

2.9. Immunoprotection assay

Zebrafish were grouped into the immunized groups and unimmunized control group. One of the immunized group was i.p. immunized with formalin-treated A. hydrophila (1 × 10⁶ CFU/fish). The other immunized group was i.p. injected with the same dosage of formalin-treated A. hydrophila vaccine plus administration of anti-Cd154 Ab (5 μg/fish) and sCd40-Ig (10 μg/fish) [29]. As control, another immunized group was administered with formalin-treated A. hydrophila (1 × 10⁵ CFU/fish) vaccine plus administration of mouse IgG (5 μg/fish) or hIgG1Fc (10 μg/fish). After immunization for 35 days, the zebrafish in all the groups were challenged with living A. hydrophila (1 × 10⁵ CFU/fish). Each group was monitored every 8 h and the mortality was recorded. The statistics of survival were analyzed according to the ratio of the surviving zebrafish numbers at the corresponding time to the total quantity.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The titers of antigen-specific IgZ and IgM Abs were measured by indirect ELISA as previously described [30]. Briefly, TcpAh protein (5 μg/ml) was used to coat 96-well plates with 100 μl/well overnight at 4 °C. The wells were washed thrice with PBST (PBS with 0.05% Tween-20) and blocked with 2% BSA (200 μl/well) of PBST at 37 °C for 1 h. Afterward, the serum or mucus (diluted at different ratios) was added (200 μl/well) and incubated at 37 °C for 2 h. Following washing, mouse anti-IgZ Ab or mouse anti-IgM Ab or mouse IgG (100 μg/ml, 100 μl) was added to each well for 2 h at 37 °C. Then, the plates were washed thrice, incubated with HRP-conjugated goat anti-rabbit IgG Ab (1:3,000, 100 μl/well) for 1 h at 4 °C and washed again with PBST for three times with PBST for 3 min. Then, 200 μl of 3,3’,5’,5’-Tetramethylbenzidine (P0209; Beyotime) was added to each well, incubated at room temperature in the dark for 15–20 min until the color was developed. The reaction was stopped with 2 M H₂SO₄ and measured at 450 nm on a Synergy H1 Hybrid Reader (BioTek Instruments). Ab titer is defined as the highest serum or mucus dilution at which the A450 ratios (A450 of post-immunization sera/A450 of pre-immunization sera) is greater than 2.1.

2.11. Statistical analysis

For an analysis of the differences between groups, data were performed using an unpaired two-tailed t-test by GraphPad Prism 6.0. The results were expressed as mean ± SD based on at least three independent experiments. For survival curve analysis, log-rank test was used by SPSS 21.0 with the p values shown. The number of fish samples in each group exceeded 30. Significant difference was considered at p < 0.05 (*p < 0.05, **p < 0.01, and ***p < 0.001).

3. Results

3.1. Preparation of polyclonal antibody and recombinant proteins

For functional investigations of Cd154 and its association with Cd40, we produced the mouse anti-Cd154 polyclonal antibody (Ab) and the recombinant soluble Cd154 protein (sCd154), the soluble Cd40 protein fused with a Fc fragment of human IgG1 isotype (sCd40-Ig) and the human IgG1 Fc protein (hIgG1Fc) in E.coli. The sCd154 and sCd40-Ig proteins contain the extracellular regions of the Cd154 and Cd40 membrane proteins with an expected molecular weight of 28 kDa and...
56 kDa, respectively, in which the scD40-Ig protein was fused with a Fc domain of human IgG-Fc fragment for the purpose of promoting the dimerization of scD40 proteins. The validity and specificity of the anti-Cd154 Ab were determined by Western blot analysis. The results showed that the mouse anti-Cd154 Ab showed high specificity to both recombinant scD154 protein expressed in E. coli, recombinant Cd154 protein expressed in eukaryotic system (HEK293T cells) and the naturally expressed Cd154 protein derived from zebrafish leukocytes, with an average titer above 1:8,000 as detected by ELISA in which the plate was coated with the E. coli-expressed scD154 proteins. In this case, the anti-Cd154 Ab was clearly combined with the target Cd154 proteins with a molecular weight of 28 kDa (for E. coli-expressed scD154), and 30 kDa (for HEK293T cell-expressed Cd154 protein and leukocyte-derived Cd154), respectively (Fig. S1B). In addition, the recombinant proteins were separated by the affinity column, and the molecular weight and purity of these proteins were assessed through 12% SDS-PAGE followed by Coomassie brilliant blue R250 staining. The result showed that the antibody of human IgG-Fc, scD154 and scD40-Ig proteins were clearly detected with an expected the molecule weight of 26 kDa, 28 kDa and 56 kDa, respectively (Fig. S1A).

3.2. Involvement of Cd40 and Cd154 in IgZ-mediated antibacterial defense

To provide initial insights into the functional role of Cd40 and Cd154 in IgZ-mediated antibacterial, the induced-expressions of cd40 and cd154 in response to A. hydrophila infection were examined in various immune-related tissues, particularly mucosa-associated lymphoid tissues (MALTs), including gill-, skin- and gut-associated lymphoid tissues. The results showed that the expression levels of cd40 and cd154 were significantly upregulated (p < 0.05) upon A. hydrophila stimulation in all the tissues examined, including head kidney, spleen and the various MALTs, which was accompanied with the remarkable increase in igz expression (Fig. 1A, 1B and 1C). This outcome suggested the role of Cd40 and Cd154 in IgZ production by activating IgZ+ B cells via interaction between the potential Cd40 IgZ+ B cells and Cd154+ T cells. In accordance with this hypothesis, the Cd40 was clearly located on the IgZ+ B cells sorted from the leucocytes of selected lymphoid tissues as determined by immunofluorescence staining, which suggesting the real existence of Cd40+ IgZ+ B cell subset (Fig. 1D).

For clarification, an in vivo depletion assay was performed to assess the role of Cd40-Cd154 interaction in the activation of Cd40+ IgZ+ B cells by depleting Cd154+ cells via i.p. administering anti-Cd154 Ab (Fig. S1C, S1D, and S1E). As expected, upon stimulation with A. hydrophila, the percentage of Cd40+ IgZ+ B cells was significantly declined (p < 0.05) in the anti-Cd154 Ab-administered groups compared with that of the non-related IgG isotype-treated control groups, as shown by the changes of percentage from 33.00 ± 1.56% to 1.71 ± 0.42% in peripheral blood leucocytes (PBLs), from 15.80 ± 1.13% to 7.95 ± 1.69% in gill, from 64.73 ± 2.57% to 18.87 ± 1.33% in skin, from 37.95 ± 1.77% to 8.02 ± 0.68% in gut, and from 15.20 ± 0.96% to 6.38 ± 1.43% in head kidney, respectively (Fig. 2A, B). In addition, an in vivo blockade assay was conducted to provide further support for the regulation of Cd40-Cd154 interaction in Cd40+ IgZ+ B cell activation by...
Fig. 2. Evaluation on the functional role of Cd40/Cd154 in Cd40+ IgZ+ B cell proliferation in immune-related tissues of zebrafish with or without in vivo depletion or blockade of Cd154. (A) Examination on the percentage of Cd40+ IgZ+ B cells in different tissues by FCM analysis, in which the different treatments were presented at the top of each block diagram. Each diagram is a representative of the three independent experiments. (B, C) Histogram shows the statistical analysis of the percentage of Cd40+ IgZ+ B cells in each tissue as examined in the FCM analysis. All data were obtained from at least three independent experiments (n = 30). * p < 0.05, ** p < 0.01, *** p < 0.001.
blocking Cd154 protein on the Cd154+ cells via i.p. administering scCd40-Ig protein (Fig. S1C, S1D, and S1E). Expectedly, upon stimulation with A. hydrophila, the percentage of Cd40+ IgZ+ B cells was significantly declined (p < 0.001) in the scCd40-Ig protein-administered groups compared with that of the hlgG1Fc-treated control group, as shown by the alterations of percentage from 30.30 ± 2.07% to 4.67 ± 1.35% in PBLs, from 21.20 ± 2.62% to 5.53 ± 1.39% in gill, from 40.03 ± 1.67% to 15.05 ± 1.63% in skin, from 38.40 ± 2.78% to 7.16 ± 0.41% in gut, and from 23.77 ± 2.60% to 4.08 ± 1.20% in head kidney, respectively (Fig. 2A, C). Altogether, the depletion and blockade assays indicated that the Cd40-Cd154 interaction plays a crucial regulatory role in IgZ-mediated antibacterial defense.

Next, an immunoprotection assay was performed to evaluate the costimulatory regulation of Cd40-Cd154 interaction in vaccine-initiated IgZ immunity against A. hydrophila infection. The results showed that the survival rate was significantly increased (p < 0.001) in the vaccine-immunized zebrafish group compared with that in the unimmunized zebrafish group (received mock PBS) upon challenge with the virulent A. hydrophila (Fig. 3). However, the increased survival rate of fish with vaccination was markedly impaired (p < 0.01) by the depletion of Cd154+ cells or blockade of Cd154 protein, as shown by the change of survival rate from 67.33 ± 2.51% to 45.00 ± 2.65% in anti-Cd154 Ab-administered group compared with nonrelated IgG-treated group and from 70.67 ± 2.08% to 42.33 ± 2.08% in scCd40-Ig protein-administered group compared with protein-treated group. These observations suggested the essential role of Cd40-Cd154 interaction in the activation of IgZ adaptive immunity against A. hydrophila infection initiated by vaccination.

3.3. Differential role of Cd40-Cd154 interaction in bacterial TD-/TI-antigen induced IgZ immunity

To provide deep insights into the regulatory mechanism underlying Cd40-Cd154 interaction in IgZ-mediated antibacterial immunity in response to different types of bacterial antigens, the differential functions of Cd40 and Cd154 in thymus-dependent (TD) antigen and thymus-independent (TI) antigen-induced IgZ immune responses were explored by in vivo depletion and blockade assays. For this purpose, an A. hydrophila-derived TcpAh virulence protein and an E. coli-derived LPS were used as a TD and a TI antigen, respectively. The antigen-stimulated Cd40+ IgZ+ B and Cd154+ Cd4+ T cell activation after challenging with TcpAh or LPS for 3 days were examined by flow cytometry analysis, and the antigen-specific IgZ and IgM Ab titers were determined by indirect ELISA after challenging with TcpAh or LPS for 7 and 21 days, respectively. The results showed that the TcpAh protein antigen significantly induced the IgZ Ab production at 21 days, but this reaction was markedly impaired (p < 0.001) in fish with the depletion of Cd154+ cells or blockade of Cd154 protein on the surface of Cd154+ cells, as shown by the remarkable decline (above 50%) of TcpAh-specific IgZ titers in serum and tissues compared with those of fish treated with nonrelated IgG isotype or hlgG1Fc control protein (Fig. 4A). The decline of IgZ Ab production in Cd154-deficient fish was accompanied with the inhibition of Cd40+ IgZ+ B cell proliferation (Fig. 4C and 4D). In this case, the percentage of Cd40+ IgZ+ B cells in Cd154+ cell-depleted fish was significantly declined (p < 0.001) from 26.17 ± 1.29% to 8.64 ± 1.33% in PBLs, from 17.73 ± 1.02% to 10.66 ± 1.13% in gut, from 60.47 ± 2.27% to 33.07 ± 1.66% in skin, from 31.87 ± 1.20% to 14.17 ± 1.52% in gut, and from 28.03 ± 1.55% to 7.48 ± 1.24% in head kidney, respectively. Additionally, the TcpAh antigen also significantly induced (p < 0.05) the proliferation of Cd4+ Cd154+ T cells, as shown by the percentage of Cd4+ Cd154+ T cells increased from 3.20 ± 0.17% (unstimulated control fish) to 28.90 ± 1.14% in PBLs, from 2.83 ± 0.07% to 20.56 ± 1.19% in gill, from 8.45 ± 0.78% to 29.6 ± 1.53% in skin, from 9.39 ± 1.12% to 33.50 ± 2.90% in gut, and from 3.69 ± 0.47% to 27.30 ± 1.11% in head kidney, after stimulation of fish with TcpAh protein (Fig. 5A and 5B). Importantly, the TcpAh-induced Cd4+ Cd154+ T cell proliferation was significantly inhibited (p < 0.01) by administering fish with CsA, a well-established inhibitor for T cells (Fig. 5A and 5B). Besides, the TcpAh-specific IgZ titers showed a significant decline (p < 0.01) in serum and tissues by administering fish with CsA (Fig. 5C). These observations suggested the involvement of IgZ response to bacterial TD antigens, which depends on the Cd40-Cd154 interaction between Cd40+ IgZ+ B and Cd4+ Cd154+ T cells. As a positive control, the performance of Cd40-Cd154 interaction was clearly observed in TcpAh-induced IgM (Fig. 4B and 4D) production, in which Cd40 and Cd154 were well-characterized to be a reciprocal costimulatory molecule between IgM+ B and Cd4+ T cells that were essential for the activation of TD antigen-induced IgM-mediated adaptive humoral immunity. By contrast, the LPS-induced Cd40+ IgZ+ B cell proliferation was not significantly impaired (p > 0.05) in Cd154+ cells-depleted zebrafish (Fig. 6). In this case, the percentage of Cd40+ IgZ+ B cells in tissues were comparable between anti-Cd154 Ab-administered groups and nonrelated IgG isotype-treated groups. This outcome suggested that the IgZ response to LPS is likely independent of Cd40-Cd154 interaction. Above all, it suggested that Cd40 and Cd154 may play a differential role in TD and TI antigen-induced IgZ immunity against bacterial infection.

Fig. 3. Evaluation on the functional role of Cd40 and Cd154 in vaccinated immunoprotection. (A) Depletion of Cd154+ cells or blockade of Cd154 protein impaired the vaccinated immunoprotection of zebrafish against A. hydrophila (1 × 108 CFU/fish) challenge. Kaplan-Meier survival curves represent data pooled from three independent experiments (n = 30). (B) Histogram shows the statistical analysis of the survival rate of fish (n = 30) in different treatment groups after infection with A. hydrophila for 50 h. All data were obtained from at least three independent experiments (n = 30). *p < 0.05, **p < 0.01, ***p < 0.001.
4. Discussion

Although the CD40 and CD154 homologs have been extensively identified from fish species, including zebrafish [26], flounder (Paralichthys olivaceus) [36, 37], Atlantic salmon (Salmo salar) [38], grass carp [27], Rainbow trout (Oncorhynchus mykiss) [1, 39], Nile tilapia (Oreochromis niloticus) [1], humphead snapper (Lutjanus sanguineus) [40] and small-spotted catshark (Scyliorhinus canicula) [41], the...
functional role of these two homologs in fish remains poorly understood. Previously, we found that CD40-CD154 interaction between CD4\(^+\) T and IgM\(^+\) B cells is essential for IgM Ab production in a thymus-dependent manner [26] . However, the involvement of CD40-CD154 interaction in IgZ\(^+\) B cell activation and IgZ Ab production remains unclear. The IgZ or its equivalent IgT is a fish-specific Ig class, which represents a newly discovered Ig class (named \(\zeta\) or \(\tau\)) in addition to the two previously known IgM (\(\mu\)) and IgD (\(\delta\)) classes of teleost fish. The IgZ/IgT class is extensively distributed in MALTs, including gut-, skin-, nasal-, buccal- and gill-associated lymphoid tissues (GALT, SALT, NALT, BALT and GIALT) [30, 31, 42-49]. It plays an important role in mucosal immunity against parasitic and bacterial infection, and maintaining microbiome

![Fig. 5. Validation of the thymus-dependency of TcpAh-induced IgZ immune response by using the CsA inhibitor. (A) FCM analysis for T cell activation by the change of Cd4\(^+\)Cd154\(^+\) T cell proportion in different tissues of zebrafish via in vivo stimulation with TcpAh protein and administration with CsA (5 \(\mu\)g/fish). (B) Histogram shows the statistical analysis of the percentage of Cd4\(^+\)Cd154\(^+\) T cells in each tissue as examined in the FCM analysis. (C, D) Examination on the reduced TcpAh-specific IgZ Ab and IgM Ab titers in different tissues and serum samples of zebrafish by in vivo administration of TcpAh plus CsA through ELISA. All data were obtained from at least three independent experiments (\(n = 30\)). * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).]
Thus, the functional role of IgZ/IgT is likely similar to that of IgA in mammals [30, 51]. Recently, we found that IgZ is highly expressed in zebrafish ovary, accumulates in unfertilized eggs, and is transmitted to offspring from eggs to zygotes, thereby providing new insights into IgZ acting as a maternal transfer immunoglobulin that contributes to the early defense of fish against pathogen infection [43]. In addition, we found that IgZ was also distributed in the peripheral serum besides the widely distribution in the MALTs [30]. Correspondingly, the IgZ+ B cells showed a wide distribution in the systemic primary/secondary lymphoid tissues (kidney marrow/spleen) and MALTs. The production of IgZ Ab largely depended on the CD4+ T cells, suggesting the dependency (at least partially) of IgZ immunity on primary/secondary lymphoid tissues in the systemic immune system [30, 52]. These findings suggested that the functional behavior of the IgZ/IgT family are more diverse than previously recognized. However, the molecular mechanisms underlying IgZ/IgT-mediated immunity remain to be further explored.

In the present study, we investigated the regulatory role of Cd40 and Cd154 costimulatory signals in IgZ-mediated antibacterial immunity by using an A. hydrophila-infected zebrafish model. Considering that the IgZ Ab production largely depended on the Cd4+ T cells as we previously reported [52], it seems reasonable to speculate the existence of a collaboration between Cd154-expressing Cd4+ T cells and IgZ-expressing IgZ+ B cells for initiating a TD antigen-induced IgZ immune response. In accordance with this hypothesis, the expression of cd40 and cd154 were significantly upregulated in lymphoid tissues upon A. hydrophila infection, and this upregulation was accompanied with the increased expression of igz and proliferation of Cd40+IgZ+ B cells in these tissues. Depletion of Cd154+ cells or blockade of Cd154 protein on Cd154+ cells significantly inhibited Cd40+IgZ+ B cell proliferation,

![Fig. 6. Evaluation of the functional role of Cd40 and Cd154 in TI antigen-induced IgZ immune response. (A) FCM analysis for the percentage of Cd40+IgZ+ B cells in different tissues of zebrafish by in vivo administration with LPS plus anti-Cd154 antibody. (B) Histogram shows the statistical analysis of the percentage of Cd40+IgZ+ B cells in each tissue as examined in the FCM analysis. All data were obtained from at least three independent experiments (n = 30). *p < 0.05, **p < 0.01, ***p < 0.001.](image-url)
followed by a decline in the immunoprotection of fish from *A. hydrophila* infection. For confirmation, a TcpAh virulence protein was used as a TD antigen for further examination. As expected, this TcpAh protein significantly induced IgZ adaptive immune response in a TD-antigen infection. For confirmation, a TcpAh virulence protein was used as a TD antigen. The result showed that the LPS also has an ability to induce Cd40+ IgZ+ B cell proliferation. However, the LPS-induced IgZ immune response was independent of Cd40+ Cd4+ T cells, as evidenced by the observation that Cd40+ IgZ+ B cell proliferation was not impaired in Cd40- cells-depleted fish. These results suggested that the IgZ Ab can respond to different kind of bacterial antigens. The antibacterial activity of IgZ Ab to *A. hydrophila* infection may be a consequence of combined reactions to both TD and TI antigens, including the virulence proteins (such as TcpAh) and the glycolipid endotoxins (such as LPS). The differential IgZ responses to TD and TI antigens may depend on the specialization of different IgZ+ B cell subsets derived from diverse developmental pathways in systemic primary/secondary lymphoid tissues and/or local MALTs. In this case, the TD antigens may induce IgZ response through systemic primary/secondary lymphoid-associated pathway in kidney marrow/spleen lymphoid tissues in a Cd40+ Cd4+ T cell-dependent manner; whereas, the TI antigens may induce IgZ response through MALT-associated pathway, which was independent of Cd40+ Cd4+ T cells. In fact, the expression profile of cd40/cd154 and igz in response to *A. hydrophila* stimulation was not always comparable between primary/secondary lymphoid tissues (such as spleen) and MALTs (such as gill); and this differential expression profile may partially support the above observations. In humans and mouse models, the IgM+ B cells have been classified into B1 and B2 subsets. The B-1 subset produces innate-like immune responses to the stimulation of TI-antigens of microbial pathogens, while the B2 subset succeeds long-lived memory responses via TD class-switched responses within germinal center (GC), producing affinity maturity antibodies and engaged in T cell-dependent host humoral immunity [53, 54]. Thus, whether the IgZ+ B cells consist of different subsets, such as IgZ+ B1 and IgZ+ B2 subpopulations whose performances are similar to those of IgM+ B1 and IgM+ B2 subsets, remains to be further explored. Clarification on this issue will improve the understanding for the evolutionary history of the IgZ/T family and regulatory mechanism underlying IgZ/T-mediated immunity.

In conclusion, our present study explored the regulatory role of Cd40 and Cd154 costimulatory signals in IgZ-mediated immune defense against bacterial infection. The results highlighted the differential role of Cd40/Cd154 interaction in bacterial TD and TI antigen-induced IgZ immunity, and provided new insights into the existence of diverse regulatory mechanisms underlying IgZ-mediated antibacterial immune reactions. This study also improved the current understanding of the coevolutionary relationship between IgZ/IgT immunoglobulins and CD40/Cd154 costimulatory molecules.

**Author’s contribution**

Ning Su: Conceptualization, Methodology, Materials preparation, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. Chong-bin Hu: Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. Tong Shao: Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. Hao Li: Formal analysis, Data curation. Chun-yu Jin: Materials preparation, Formal analysis. Ding-Dong Fan: Materials preparation. Ai-fu Lin: Data curation. Li-xin Xiang: Supervision, Project administration, Data curation, Writing – original draft, Writing – review & editing. Jian-zhong Shao: Conceptualization, Methodology, Supervision, Project administration, Data curation, Writing – original draft, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2021.100038.

**References**

1. B. Li, Y. Li, S. Wu, Y. Yang, S. Fu, X. Yin, X. Tu, L. Fang, Z. Guo, J. Ye, Identification and functional characterization of CD154 in T cell-dependent immune response in Nile tilapia (Oreochromis niloticus), Fish Shellfish Immunol. 111 (2021) 102–110.

2. A.G. Pose, E.S. Rodrigues, A.C. Mírdez, J.N. Gómez, A.V. Redondo, E. R. Rodríguez, E.M. Ramos, A. Gutiérrez, M.P. Moltó, D.G. Roche, Y.S. Ugalde, A. M. López, Dual function of the hemagglutinin H5 fused to chicken CD154 in a potential strategy of DIVA against avian influenza disease: preliminary study, Open Vet. J. 5 (2) (2015) 138–147.

3. O. Sanchez Ramos, A. Gonzalez Pose, S. Gomez-Puerta, J. Noda Gonzalez, A. Vega Redondo, J.C. Aguilera Benites, L. Suarez Amaran, N.C. Parra, J.R. Toledo Alonso, Avian CD154 enhances humoral and cellular immune responses induced by an adenosine vector-based vaccine in chickens, Comp. Immunol. Microbiol. Infect. Dis. 34 (3) (2011) 259–265.

4. J. Oh, N. Wu, A.J. Barczak, R. Barbeau, D.J. Erle, J.S. Shin, CD40 mediates maturation of thymic dendritic cells driven by self-reactive CD4(-) thymocytes and supports development of natural regulatory T cells, J. Immunol. 200 (4) (2018) 1399–1412.

5. A. Javmen, H. Szmacinski, J.R. Lakowicz, V.Y. Tochshakov, Blocking TIR domain interactions in TLR9 signaling, J. Immunol. 201 (3) (2018) 995–1006.

6. C. van Kooten, J. Banchereau, CD40-CD40 ligand, J. Leukoc. Biol. 67 (1) (2000) 2–17.

7. M. Croft, C.A. Benedict, C.F. Ware, Clinical targeting of the TNF and TNFR superfamilies, Nat. Rev. Drug Discov. 12 (2) (2013) 147–166.

8. U. Schonbeck, F. Mack, P. Libby, CD154 (CD40 ligand), Int. J. Biochem. Cell Biol. 32 (7) (2000) 687–693.

9. A. Miga, S. Masters, M. Gonzalez, R.J. Noelle, The role of CD40-CD154 interactions in the regulation of cell mediated immunity, Immunol. Invest. 29 (2) (2000) 111–114.

10. E.A. Clark, J.A. Ledbetter, Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50, Proc. Natl. Acad. Sci. U. S. A. 83 (12) (1986) 4494–4496.

11. S. Paule, B. Blin-Henriknson, H. Mellerstedt, K. Hoho, H. Ben-Aissa, P. Perlmann, A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes, Cancer Immunol. Immunother. 20 (1) (1985) 23–28.

12. A. Durandy, S. Kracker, Immunoglobulin class-switch recombination deficiencies, Arthritis Res. Ther. 14 (4) (2012) 218.

13. T. Zhang, R.N. Piersø, A.M. Arima-zadeh 3rd, Update on CD40/Cd154 blockade in transplant models, Immunotherapy 7 (8) (2015) 899–911.

14. M.E. Munroe, G.A. Bishop, A costimulatory function for T cell CD40, J. Immunol. 167 (2001) 671–682.

15. A.W. Tong, M.J. Stone, Prospects for CD40-directed experimental therapy of human cancer, Cancer Gene Ther. 10 (1) (2003) 1–13.

16. M.E. Munroe, G.A. Bishop, A costimulatory function for T cell CD40, J. Immunol. 167 (2001) 671–682.

17. T. Chand Dakal, B. Dhabhai, D. Agarwal, R. Gupta, G. Nagda, A.R. Meena, A. R. Dhakar, A. Menon, R. Mathur, Y. Yadav Mona, A. Sharma, Mechanistic basis of co-stimulatory CD40-CD40L ligation mediated regulation of immune responses in cancer and autoimmune disorders, Immunobiology Immunobiology (2019), 151899.

18. I.R. Ferrer, D. Liu, D.F. Pinelli, B.H. Koehl, L.A. Stempora, M.L. Ford, CD40/Cd154 blockade inhibits dendritic cell expression of inflammatory cytokines but not costimulatory molecules, J. Immunol. 189 (9) (2012) 4387–4395.

19. A. Brzoni, H.D. Ochs, The hyper IgM syndrome—an evolving story, Pediatr. Res. 56 (4) (2004) 519–525.

20. J.L. Karnell, S.A. Rieder, R. Etinger, R. Kolbeck, Targeting the CD40/Cd40L pathway in autoimmune diseases: humoral immunity and beyond, Adv. Drug. Deliv. Rev. 141 (2019) 92–103.
[20] I.N. Bulitsaioarv, H. Lum, G. Berke, D.M. Paulnock, P.M. Sondel, A.L. Rakhmilevich, CD40 ligation activates murine macrophages via an IFN-gamma-dependent mechanism resulting in tumor cell destruction in vitro, J. Immunol. 174 (10) (2005) 6013–6022.

[21] M.D. Szemans, P. Kelemen, M. Zeyda, G. Bohmig, G. Stoffer, G.J. Zlabinger, CD40 triggered human monocyte-derived dendritic cells convert to tolerogenic dendritic cells when AK3 activity is inhibited, Transplant. Proc. 34 (5) (2002) 1407–1408.

[22] D.Y. Ma, E.A. Clark, The role of CD40 and CD154/CD40L in dendritic cells, Semin. Immunopathol. 21 (5) (2009) 265–272.

[23] C. Ziske, P.F. Ettzrod, A.S. Eliz, M. Gorschützer, J. Strebl, D. Flieger, D. Messmer, V. Schmitz, M.A. Gonzalez-Cartona, E. Sirvers, P. Brossart, T. Sauerbruch, L. G. Schmidt-Wolf, Increase of in vivo antitumoral activity by CD40L. (CD154) gene transfer into pancreatic tumor cell-dendritic cell hybrids, Pancreas 38 (7) (2009) 758–765.

[24] B.J. Hamilton, A. Genin, R.Q. Cron, W.F. Rigby, Delineation of a novel pathway for IgM immune response to Ishchihoforhodin-11c and role of IgG2, Mol. Cell. Biol. 23 (2) (2003) 510–525.

[25] A.D. Straw, A.S. MacDonald, E.Y. Denkers, E.J. Pearce, CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses, J. Immunol. 170 (2) (2003) 727–734.

[26] Y.F. Gong, L.X. Xiang, J.Z. Shao, CD154-CD40 interactions are essential for thymus-dependent antibody production in zebrafish: insights into the origin of costimulatory pathway in helper T cell-regulated adaptive immunity in early vertebrates, J. Immunol. 182 (12) (2009) 7749–7762.

[27] A.B. Lu, Y.X. Chen, Z.W. Cui, X.Y. Zhang, L.F. Lu, S. Li, X.Q. Xia, P. Nie, Y.A. Zhang, Characterization of grass carp CD40 and CD154 genes and the association between their polymorphisms and resistance to grass carp reovirus, Fish Shellfish Immunol. 81 (2018) 304–308.

[28] H.P. Tang, C. Huang, C.B. Hu, H. Li, T. Shao, J.F. Ji, B. Dai, D.D. Fan, A.F. Lin, L. X. Shao, Inhibitory role of an aeronasom hydrophilic TIR domain effector in antibacterial immunity by targeting TLR signaling complexes in zebrafish, Front. Microbiol. 12 (2021), 694081.

[29] T. Shao, W. Shi, J.Y. Zheng, X.A. Xu, A.F. Lin, L.X. Xiang, J.Z. Shao, Costimulatory function of CD58/Cd2 interaction in adaptive humoral immunity in a zebrafish model, Front. Immunol. 9 (2018) 1204.

[30] J.F. Ji, C.B. Hu, T. Shao, D.D. Fan, N. Zhang, A.F. Lin, L.X. Xiang, J.Z. Shao, Differential immune responses of immunoglobulin Z subclass members in antibacterial immunity in a zebrafish model, Immunology 162 (1) (2021) 105–120.

[31] H.Y. Xu, F. Dong, X. Zhai, K.F. Meng, G.K. Han, G.F. Cheng, B.Z. Wu, N. Li, Z. Xu, Characterization of mucosal immunoglobulin IgT in the gills of rainbow trout (Oncorhynchus mykiss) after infection with Flavobacterium columnare, Fish Shellfish Immunol. 99 (2020) 654–662.

[32] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Genderson Jorgensen, S.E. LaPatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728.

[33] I. Salinas, R. Musharrafieh, E.T. Larragoite, K. Crossey, E.B. Erhardt, S.A.M. Martin, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity in teleost fish, Dev. Comp. Immunol. 35 (12) (2011) 1346–1365.

[34] Z. Xu, D. Parra, D. Gómez, I. Salinas, Y.A. Zhang, L. von Genderson Jorgensen, R. D. Heinecke, K. Buchmann, S. LaPatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U. S. A. 110 (32) (2013) 13097–13102.

[35] L. Tacchi, R. Musharrafieh, E.T. Larragoite, K. Crosse, E.B. Erhardt, S.A.M. Martin, S.E. LaPatra, I. Salinas, Nasal immunity is an ancient arm of the mucosal immune system of vertebrates, Nat. Commun. 5 (2014) 5205.

[36] Y.Y. Yu, W. Kong, Y.X. Yin, F. Dong, Z.Y. Huang, G.M. Yin, S. Dong, I. Salinas, Y. A. Zhang, Z. Xu, Mucosal immunoglobulins protect the olfactory organ of teleost fish against parasitic infection, PLoS Pathog. 14 (11) (2018), e1007251.

[37] F. Dong, G.M. Yin, K.F. Meng, H.Y. Xu, X. Liu, C.Q. Wang, Z. Xu, IgT plays a predominant role in the antibacterial immunity of rainbow trout olfactory organs, Front. Immunol. 11 (2020), 583740.

[38] Y.A. Zhang, I. Salinas, J.L. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (9) (2010) 827–835.

[39] F. Wang, C.B. Hu, J.X. Ma, K. Gao, L.X. Xiang, J.Z. Shao, Characterization of γδ T cells from zebrafish provides insights into their important role in adaptive humoral immunity, Front. Immunol. 7 (2016) 675.

[40] N. Baumgarth, A hard(y) look at B-1 cell development and function, J. Immunol. 199 (10) (2017) 3387–3394.

[41] L.Y. Zhu, T. Shao, L. Nie, L.Y. Zhu, L.X. Xiang, J.Z. Shao, Evolutionary implication of B-1 lineage cells from innate to adaptive immunity, Mol. Immunol. 69 (2016) 123–130.