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ORIGINAL ARTICLE

**Hsin-Wei Chen · Hsing-I Huang · Yi-Ping Lee
Li-Li Chen · Hsiung-Kun Liu · Mei-Lien Cheng
Jy-Ping Tsai · Mi-Hua Tao · Chou-Chik Ting**

Linkage of CD40L to a self-tumor antigen enhances the antitumor immune responses of dendritic cell-based treatment

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Abstract CD40–CD40 ligand (CD40L) interaction is an important costimulatory signal in the interaction between T cells and antigen-presenting cells (APC). In the present study, we determined whether the linkage of CD40L to the tumor-specific idiotype (Id) derived from a murine B-cell lymphoma, 38C13, could enhance its immunogenicity when presented by dendritic cells (DC). We showed that bone marrow-derived DC pulsed with Id-CD40L upregulated the expression of CD40, CD80, CD86, and major histocompatibility complex (MHC) class II molecules with the increased production of interleukin-12 (IL-12). Mice immunized with DC loaded with Id-CD40L showed high levels of anti-Id antibody response of both IgG2a and IgG1 isotypes. In addition, nylon wool-enriched T cells from these immunized mice showed a tumor-specific T-cell proliferative response upon stimulation with Id protein. Mice immunized with DC pulsed with Id alone failed to show any of these immune responses. Immunization with DC pulsed with Id-CD40L showed increased resistance to the challenge by 38C13 tumor, and tumor growth was significantly retarded. Together, these results show that linkage of CD40L to a self-tumor antigen enhances the anti-tumor immune response in DC-based treatment.

H.-W. Chen · Y.-P. Lee · L.-L. Chen · H.-K. Liu
M.-L. Cheng · J.-P. Tsai · C.-C. Ting (✉)
Immunology Group, National Health Research Institutes,
Taipei, Taiwan
E-mail: gting@nhri.org.tw
Tel.: +886-2-28741271
Fax: +886-2-28733664

H.-I. Huang · M.-H. Tao
Institute of Biomedical Sciences, Academia Sinica,
Taipei, Taiwan

C.-C. Ting
Cooperative Laboratory, Veterans General Hospital,
National Health Research Institutes. 201,
Shih-Pai Road, Sec. 2, Taipei, Taiwan

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Introduction

B lymphocytes express unique idiotypes (Id) resulting from the immunoglobulin gene rearrangements that are involved in antibody production. Since each B-cell clone gives rise to a lymphoma uniquely expressing this Id, it can serve as a specific cancer antigen. However, a major obstacle is that autologous Id protein is poorly immunogenic. It must be conjugated to a highly immunogenic exogenous carrier protein, for example keyhole limpet hemocyanin (KLH), to increase the immunogenicity of the tumor idiotype protein [24]. Alternatively, Id protein can be genetically fused with cytokine molecules, including granulocyte–macrophage colony-stimulating factor (GM–CSF), interleukin-2 (IL-2), or IL-4 to enhance the anti-Id immune response [9, 28, 44]. Another strategy is to fuse Id with cytotoxic T-lymphocyte antigen 4 (CTLA-4) to specifically target the fusion protein to antigen-presenting cells (APC) through CTLA-4 and CD80–CD86 interaction [22].

Recently, the ability of dendritic cells (DC) to generate antitumor immune responses has been documented in a variety of tumor models. These promising results include several approaches to arm DC. For example, DC was transduced by viral vectors encoding tumor antigens [10, 15] or pulsed with tumor lysates [26, 29], synthetic peptides [8, 32, 47], RNA [1, 4, 19], and proteins [34, 45]. However, the optimal form of tumor antigen for DC loading still remains an unresolved issue. Here, we examined an Id-CD40 ligand (Id-CD40L) fusion protein linking tumor antigen to CD40 ligand (CD40L) for efficient DC loading.

The rationale for using Id-CD40L was based on the fact that CD40 and CD40L interactions play a wide role in the regulation of immune responses [16]. Our recent data have shown that in vivo immunization of Id-CD40L fusion protein significantly enhances the

anti-Id antibody response and results in potent anti-tumor immunity [21]. Recent studies demonstrate that CD40–CD40L interaction is important for various DC functions in vitro and in vivo. Interaction of CD40 and CD40L in vivo regulates the migration of antigen-bearing DC from the skin to draining lymph nodes and plays a vital role in the initiation of acquired T-cell-mediated immunity [33]. CD40 and CD40L interaction triggers an important activating signal to APC [17, 42], as demonstrated by the upregulation of CD80 and CD86 which are involved in T-cell costimulation. Furthermore, ligation of CD40 with CD40L on the surface of DC regulates the production of cytokines such as IL-8, tumor necrosis factor alpha (TNF-α) and IL-12 [6, 7, 27]. Production of IL-12 by DC in particular is critical for promoting T helper 1 (Th1) cell maturation and the development of cell-mediated immunity. In addition to enhancing DC function, CD40–CD40L interaction may counteract the effect of other factors which may be harmful to DC. For instance, DC function could be suppressed by IL-10 [12, 36, 37, 40, 41]. Therefore, IL-10 secretion by tumor cells has been shown to be one of the mechanisms by which tumor cells can escape immunological recognition and destruction [25, 35, 43]. CD40–CD40L interaction can abrogate the IL-10-mediated inhibition of DC function [5]. It has also been demonstrated that the interactions between CD40 and CD40L can prevent tumor-induced DC apoptosis [13]. Thus, these studies suggest that targeting tumor antigen to DC through CD40–CD40L interaction may provide a new strategy for enhancing DC potency.

We therefore sought to determine whether DC pulsed with Id-CD40L fusion protein could enhance their potency for initiation of the immune responses. In this study, we show that Id-CD40L fusion protein is very efficient in activating bone marrow-derived DC by upregulating the costimulatory molecules and the production of IL-12. In addition, loading DC with Id-CD40L has been successfully used for generating in vivo Id-specific immune responses, and has induced the retardation of tumor growth.

Materials and methods

Mice and cell lines

Female 6- to 8-week old C3H/HeN mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). All animals were housed at the laboratory animal facility (Taipei Veterans General Hospital, Taipei, Taiwan). All of the studies were approved by the animal committee of the National Health Research Institutes, and were performed according to their guidelines. The carcinogen-induced 38C13 B-cell lymphoma has been described previously [3] and was a gift from R. Levy (Stanford University, Stanford, Calif.).

Production and purification of idiotype fusion proteins

The construction of Id-CD40L has been described previously [21]. Transfectomas expressing Id, Id-GM–CSF or Id-CD40L were expanded for large-scale production in RPMI containing 1% low IgG fetal calf serum (HyClone Laboratories, Logan, Utah). Supernatant was collected and purified by protein A chromatography as previously described [44]. Endotoxin contents of the purified proteins were determined by chromogenic limulus amebocyte lysate test kit (BioWhittaker, Walkersville, Md.) according to the manufacturer's instructions. All of the purified proteins contained less than 0.3 endotoxin IU/μg protein (data not shown).

Generation and antigen loading of bone marrow-derived DC

Bone marrow-derived DC were prepared as previously described [23] with some modifications. Briefly, after removing muscle tissues from the femurs and tibias, bone marrow was flushed and depleted of red cells by ammonium chloride lysis. After washing, bone marrow cells were depleted of T, B, and MHC class II^+ cells by a cocktail of monoclonal antibodies (mAb) and rabbit complement for 30 min at 37°C. The mAb used were GK1.5 (anti-CD4), 53–6.7 (anti-CD8), RA3–3A1 (anti-B220), and M5/114 (anti-MHC class II) (TIB-207, TIB-105, TIB-146, and TIB-120, respectively; American Type Culture Collection, Manassas, Va.). Cells (1×10^6) were place in 24-well plates in 1 ml of medium supplemented with 500 IU/ml recombinant murine GM–CSF and 1,000 IU/ml recombinant murine IL-4. Medium was replenished after two days by gently swirling the plates, aspirating the medium, then adding fresh medium with cytokines. After four days of culture, nonadherent and loosely adherent cells were harvested by gently pipetting and replated at 1×10^6 cells/ml in fresh medium supplemented with 500 IU/ml recombinant murine GM–CSF and 3,000 IU/ml recombinant murine IL-4. Medium with cytokines was replenished on the sixth day, cells were cultured overnight in the presence of medium alone or the tested Id proteins. The following day, nonadherent and loosely adherent DC were harvest and washed, then resuspended in phosphate-buffered saline (PBS) for immunization.

Flow cytometry analysis

To determine the phenotype of bone marrow-derived DC, cells were cultured for five days then pulsed with Id or Id-CD40L fusion protein overnight. Forty-eight hours later, cells were used for staining. Nonspecific staining was blocked by incubation with rat anti-mouse CD16/CD32 antibody (2.4G2, Pharmingen, San Diego, Calif.) in PBS for 10 min at 4°C. The following commercially available mAb were used (all purchased from Pharmingen, San Diego, Calif.) and were conjugated with fluorescein isothiocyanate or phycoerythrin: anti-CD40 (3/23), anti-CD80 (16–10A1), anti-CD86 (GL1) and anti-$I\text{-}A^k$ (11–5.2). After washing, cells were collected and analyzed on a FACStar Plus (Becton Dickinson) instrument with Cellquest software.

Measurement of IL-12 levels

Cultured bone marrow-derived DC were stimulated with Id-CD40L fusion protein. Supernatants were collected 24 h later, centrifuged and stored at –80°C. Quantitative determination of total mIL-12 (p70) was measured by IL-12 enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, Minn.) according to the manufacturer's instructions and expressed in pg/ml.

Immunization

For DC immunization, DC cultured for six days were pulsed with Id fusion proteins at 100 nM overnight before injection. Washed antigen-pulsed DC were injected intravenously (i.v.) into tail veins of C3H/HeN mice. Two weeks later, mice were boosted with the same dosage. Each immunization contained 1×10^5 DC in 0.2 ml of PBS. For some experiments, animals were immunized once only.

Antibody assay

One week after the booster immunization, sera samples were collected by tail bleeding. Anti-Id IgG levels in the immune sera were determined by titering sera on ELISA plates coated with purified 38C13 Id. Bound IgG were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc (ICN Pharmaceuticals, Ohio). Color was generated by adding 2,2'-azino-*bis*-(ethylbenzthiazoline sulfonic acid) (Sigma Chemical), and absorbance at 405 nm was measured on an ELISA reader. For measurement of IgG anti-Id antibodies, the readings were taken with reference to a purified monoclonal anti-38C13 Id antibody. For measurement of IgG1 and IgG2a anti-Id isotypes, biotin-conjugated rat anti-mouse IgG1 (A85–1; Pharmingen, San Diego, Calif.) and IgG2a (R19–15; Pharmingen, San Diego, Calif.) were used as detectors. Streptavidin–HRP conjugate (Pharmingen) was then added. Color was developed as described above. End-point titers were defined as the highest serum dilution that resulted in an absorbance value greater than 0.1.

Assays for lymphocyte proliferation and cytokine secretion

To determine whether Id-specific lymphoproliferative responses were induced in immunized animals, spleens were removed 1 week after immunization to make single-cell suspensions.

Nylon wool-enriched T cell were prepared, 0.5 ml of nylon wool-enriched splenic T cells at 4×10^6 cells/ml were mixed with 0.5 ml of naive splenocytes (irradiated with 2,000 rad) at 2×10^6 cells/ml and were added to each well of a 24-well plate. They were further primed with 38C13 Id protein at 20 μg/ml. After 4 days culture, 0.1 ml of the cultured cells were dispensed into a 96-well flat bottomed plate in triplicate, and were pulsed with ^{3}[H]-thymidine for 6 h. The Δ cpm was calculated as the mean cpm of the stimulated wells subtracted from the mean cpm of the control wells (receiving cells only).

To measure IFN-γ and IL-4 secretion, nylon wool-enriched T cells were cultured and pulsed with 38C13-Id protein as described above. After culturing for 3 or 4 days, cell-free supernatants were harvested and stored at –80°C. Quantitation of total IFN-γ or IL-4 was determined by IFN-γ or IL-4 ELISA kits (R&D Systems) according to the manufacturer's instructions and expressed in pg/ml.

Tumor challenge

Mice were challenged subcutaneously with 500 38C13 cells 2 weeks after the booster immunization. Tumor size was assessed three times a week and the animals were killed when the tumor reached 3,000 mm^3 in size. Tumor volume was calculated by the formula $V=\pi\times abc/6$, where a, b and c are the orthogonal diameters. Statistical analysis was performed by the Student's t-test. Data were considered statistically significant at $P \le 0.05$.

Results

CD40L fusion protein activates DC to upregulate costimulatory molecule expression and IL-12 production

CD40–CD40L interactions play a pivotal role in the regulation of the immune response. An important role of this interaction is to activate the APC to upregulate their costimulatory function and cytokine production [17, 18, 46]. In this study, we investigated whether immunogenicity could be augmented by employing Id-CD40L fusion protein in DC-based vaccine. The Id-CD40L fusion protein is composed of 38C13 Id and human IgG1, κ constant region with the extracellular domains of murine CD40L attached at the carboxy-terminal end of each of the immunoglobulin heavy chains [21]. To analyze the functional activity of different fusion proteins, DC were pulsed with Id, Id-GM–CSF or Id-CD40L at 200 nM on day 5. The expression of costimulatory molecules on DC was analyzed by flow cytometry on day 7. As shown in Fig. 1A, Id-CD40L fusion protein significantly enhanced the expression of CD40, CD80, CD86, and MHC II molecules compared with Id, Id-GM–CSF or unpulsed DC control. In contrast, DC pulsed with Id or Id-GM–CSF expressed similar levels of costimulatory molecules as the medium control, except for a slight increase of CD40 expression after pulsing with Id-GM–CSF. At a lower concentration (12.5 nM), Id-GM–CSF did not have any effect on the expression of these molecules (data not shown). Upon further titration, the effect of Id-CD40L at three different concentrations (200, 20 and 2 nM) was determined, as shown in Fig. 1B. Id-CD40L fusion protein even at a low concentration of 20 nM significantly enhanced the expression of CD40, CD80, CD86, and MHC II molecules compared with the unpulsed DC control. This effect became negligible at 2 nM.

We then examined whether Id-CD40L fusion protein induced IL-12 production by bone marrow-derived DC. Bone marrow cells were cultured, then pulsed with a variety of fusion proteins on day 5, as described above. One day later, the supernatants were harvested and analyzed for the presence of IL-12 p70 by ELISA. We found that Id-CD40L fusion protein significantly induced IL-12 production in a dose-dependent fashion (Fig. 2). In contrast, pulsing with Id or Id-GM–CSF was unable to induce detectable levels of IL-12, even at concentrations up to 200 nM. These results indicate that Id-CD40L fusion protein retains the ability to activate bone-marrow-derived DC, and thus might be effective in eliciting antitumor immune responses.

DC pulsed with Id-CD40L fusion protein induce humoral and cellular anti-Id immune responses

Since Id-CD40L treatment enhanced DC for the production of costimulatory molecules and IL-12, we examined its ability to induce humoral and cellular responses by immunization of DC pulsed with Id-CD40L fusion protein. Mice were immunized twice at a biweekly interval by i.v. injection of antigen- or medium-pulsed DC, and sera were analyzed for the presence of Id-specific antibodies one week after the last immunization. DC pulsed with Id-CD40L fusion protein produced substantially higher levels of anti-38C13 Id IgG (Fig. 3A). Negligible anti-38C13 Id IgG was detected in mice immunized with DC pulsed with the Id protein alone (Fig. 3A). In addition, 89% (eight out of nine) of the animals in the Id-pulsed group remained seronegative (Id-specific titer $<1:50$), whereas all animals in the Id-CD40L pulsed group were seroconverted. There was no detectable humoral response in naive mice or mice vaccinated with DC without antigen pulsing. We then examined the isotype profiles of the anti-Id antibodies

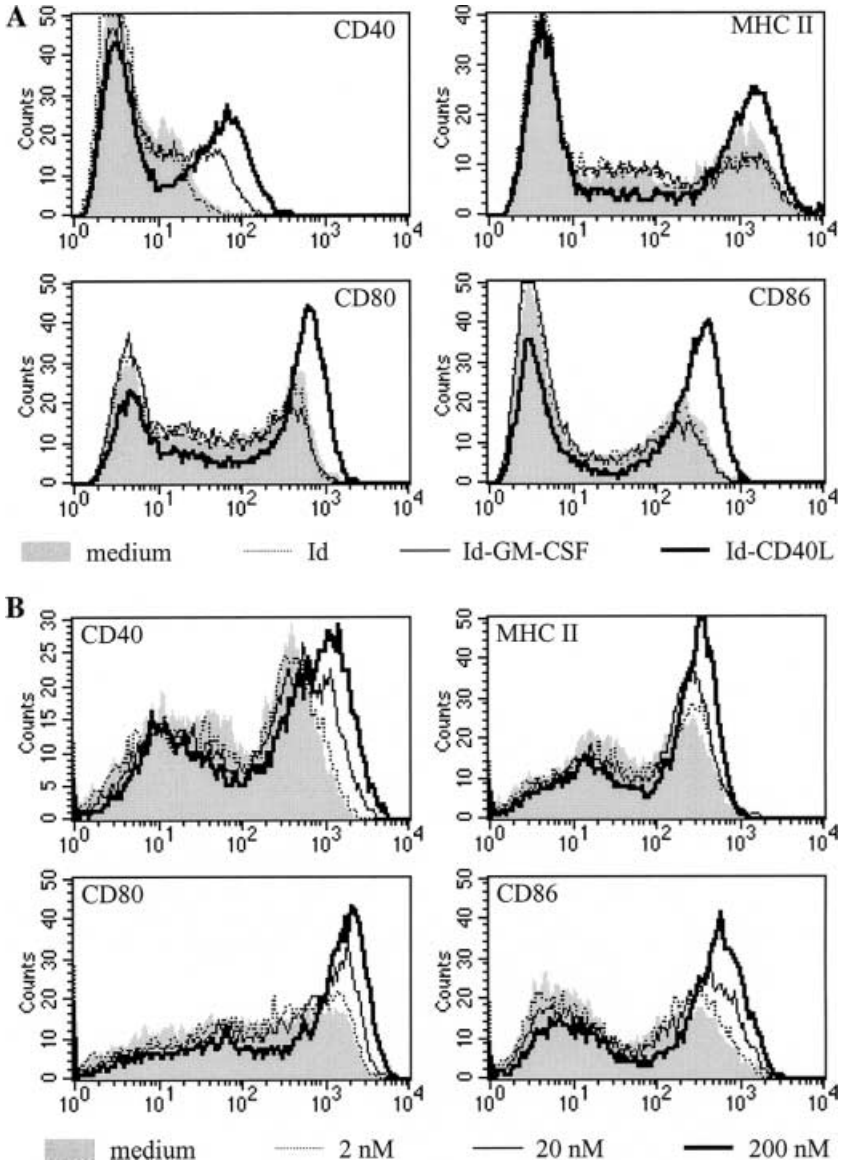


Fig. 1A, B. Bone marrow-derived DC upregulate MHC class II and costimulatory molecules after pulsing with Id-CD40L fusion protein. Bone marrow cells were cultured, and then (**A**) pulsed with Id (*dotted lines*), Id-GM–CSF (*thin lines*) or Id-CD40L (*bold lines*) fusion protein at 200 nM or unpulsed control (*filled histograms*); (**B**) pulsed with Id-CD40L fusion protein at 200 nM (*bold lines*), 20 nM (*thin lines*), 2 nM (*dotted lines*), control medium (*filled histograms*), on day 5. Costimulatory factors CD40, CD80, CD86 and MHC II were analyzed by flow cytometry on day 7. Representative results from one of two experiments are shown

induced by Id-CD40L-pulsed DC. Vaccination of mice with DC pulsed with Id-CD40L induced high titers of both IgG1 and IgG2a anti-Id antibodies (Fig. 3B).

We then examined the T-cell immune responses following vaccination of the different Id fusion proteins. Mice were immunized by i.v. injection of DC pulsed with the various Id proteins. Animals immunized with DC alone served as negative controls. One week after the immunization, splenocytes were examined for proliferation in response to specific antigen stimulation. Immunization with DC pulsed with Id-CD40L fusion protein induced a significant proliferative response to 38C13 idiotype protein (Fig. 4), while Id-pulsed DC or DC alone had no such activity. To more clearly define the Id-specific T cell response, IFN-γ production from the splenocytes of the different groups was examined (Fig. 5A). Stimulation with 38C13 idiotype protein of splenocytes from the DC–Id-CD40L group produced a substantially high level of IFN-γ (82 pg/ml), whereas stimulation with transferrin or control medium produced only low levels of IFN-γ (11 pg/ml and 11 pg/ml, respectively). In contrast, stimulation with 38C13 idiotype protein of splenocytes from the DC–Id group produced only low levels of IFN-γ (15 pg/ml), whereas stimulation with transferrin or control medium produced negligible levels of IFN-γ. There was no detectable IFN-γ secretion in the group of mice immunized with DC alone. Similar patterns were obtained for IL-4 production (Fig 5B). Taken together, these data suggest that vaccination with DC pulsed with Id-CD40L fusion protein was able to induce Id-specific antibodies and T cell responses.

Immunization with DC pulsed with Id-CD40L fusion protein is able to retard tumor growth

Given the superior anti-Id immune response elicited by vaccination with Id-CD40L-pulsed DC, we further

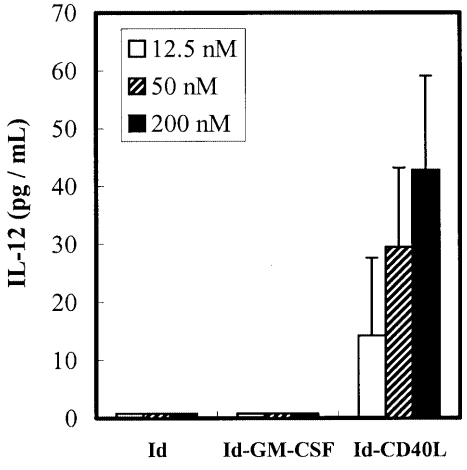


Fig. 2. IL-12 production by bone marrow-derived DC is triggered by Id-CD40L fusion protein. Bone marrow cells were pulsed with Id, Id-GM–CSF or Id-CD40L fusion protein at different concentrations after culturing for five days. Supernatants were harvested the following day. IL-12 levels of supernatants were determined by ELISA assay. Representative results from one of two experiments are shown

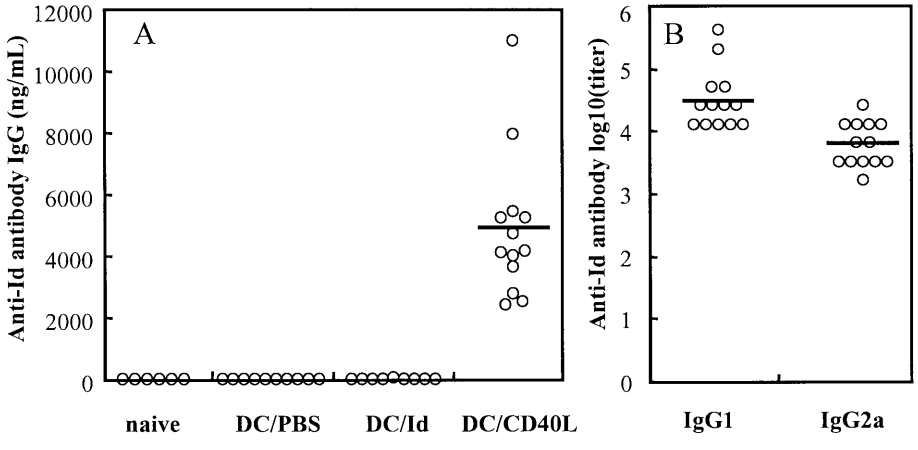
tested the immunized mice for an in vivo protective effect against challenge by 38C13 B lymphoma cells. Groups of C3H/HeN mice were given i.v. injections of antigen-pulsed DC twice at a 2-week interval. Two weeks after the last vaccination, animals were challenged with a lethal dose (500 cancer cells) of 38C13 B lymphoma cells. As shown in Fig. 6, mice immunized with DC or Id-pulsed DC were not protected, and a similar tumor progression was observed to that in the non-vaccinated animals. In contrast, mice immunized with Id-CD40L-pulsed DC displayed significantly retarded tumor growth ($P < 0.05$) when compared with other groups. These results demonstrate that immunization with Id-CD40L-pulsed DC is able to induce antitumor responses and retards tumor growth.

Fig. 3A, B. Humoral anti-Id response induced by immunization with antigen-pulsed DC. Bone marrow-derived DC were pulsed with antigen (Id or Id-fusion proteins) on day 6 at 100 nM. They were then washed the next day, and 1×10^5 DC were inoculated i.v. into 6–8-week old female C3H/HeN mice. The mice were immunized twice at a biweekly interval. One week after the second immunization, blood was drawn from the immunized mice and the anti-Id IgG antibody response (**A**) was determined by ELISA assay. Only the animals that were immunized with DC pulsed with Id-CD40L fusion protein showed significant antibody production and were analyzed for the presence of IgG1 and IgG2a anti-Id antibodies (**B**). Each *open circle* represents the antibody titer of an individual mouse. The *bar* indicates the mean antibody titer. Data are pooled from three independent experiments

Discussion

A number of barriers exist that can prevent successful cancer immunotherapy. Subsequently, most malignant tumors are incapable of inducing an adequate immune response. The immune system may perceive tumors more as harmless self- rather than as harmful signal, it may induce anergy or tolerance to tumor antigen, and tumors are thus able to escape immune surveillance, with resulting progressive growth. One of the most important tasks is to overcome these obstacles and allow the delivery of the tumor antigens in an appropriate form to be recognized by the immune system of a cancer patient. In the present study, we used B-cell lymphoma as a model system to test a new strategy for generating antitumor immunity by arming DC with fusion proteins consisting of tumor-derived Id protein and CD40L.

In our approach, DC can be successfully cultured in sufficient quantity from bone marrow. The DC phenotype was analyzed by flow cytometry. After eight days culture, these cells were 80% $CD11c^+$, 73.5% $CD40^+$, 47.4% $CD80^+$, 55.1% $CD86^+$, and 73.2% MHC class II^+ (Fig. 1; and data not shown). Our preliminary experiments indicate that the allogeneic stimulatory capacity of bone marrow-derived DC was far superior to that of splenocytes (data not shown). Bone marrow-derived DC possess the biological activity to present antigen and to induce a high level of immune response to allo-antigen. Nevertheless, C3H mice immunized with DC loaded with Id protein generated very little anti-Id antibody response (Fig. 3), and only 11% (one out of nine) of the animals underwent seroconversion. We also did not observe any in vivo antitumor immunity (Fig. 6). Lapointe et al. [30] suggested that expression of



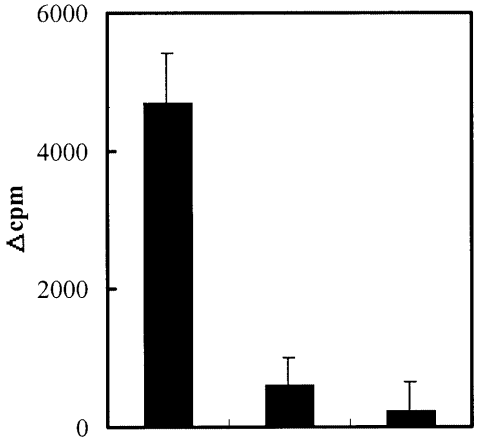


Fig. 4. Id-specific T cell responses induced by antigen-pulsed DC immunization. C3H mice were immunized with Id- or Id-CD40L-pulsed DC. Mice vaccinated with medium-pulsed DC served as controls. One week after immunization, splenocytes were pooled from two immunized mice and passed through a nylon wool column to enrich T cells. Enriched T cells were stimulated with 38C13 Id protein at 20 μg/ml and examined for their Id-specific proliferative response four days later. The Δ cpm was calculated as the mean cpm of the stimulated wells subtracted from the mean cpm of the control wells (receiving cells only). Results shown are one of three representative experiments

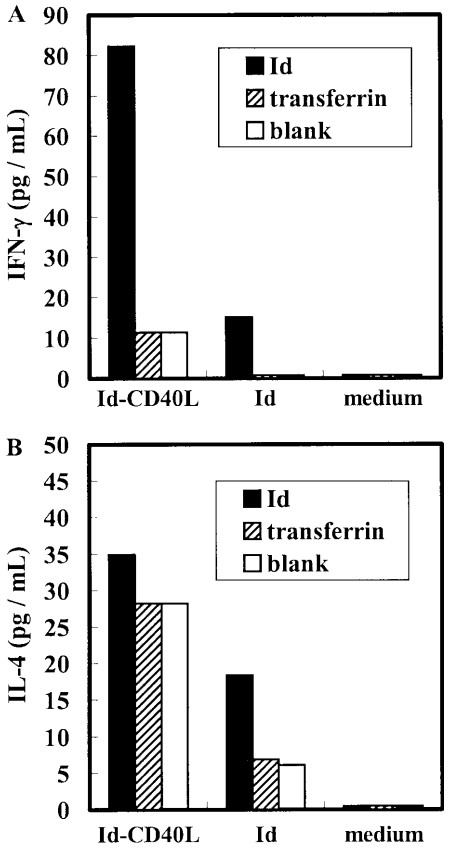


Fig. 5. IFN-γ and IL-4 secretion from T cells of vaccinated mice. Nylon wool-enriched T cells from mice immunized with DC loaded with Id, Id-CD40L, or control medium were stimulated with 38C13 Id protein at 20 μg/ml on day 7 after vaccination. The Id-specific secretion of IFN-γ (**A**) and IL-4 (**B**) was determined by ELISA assay 4 and 3 days later, respectively. Results shown are one of three representative experiments

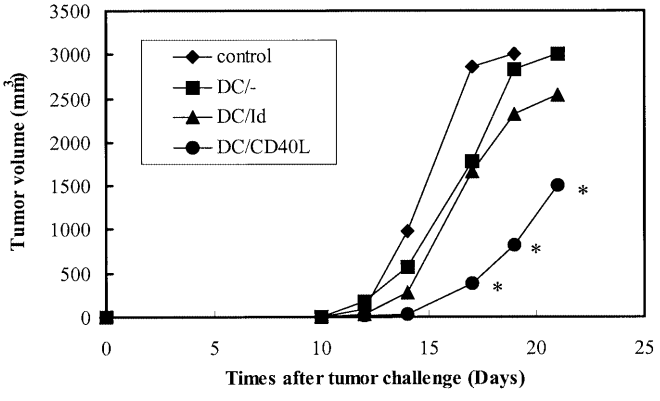


Fig. 6. Mice immunized with DC pulsing with Id-CD40L fusion protein elicited antitumor immunity. The mice were immunized as described in Fig. 3. Two weeks after the last immunization, they were then challenged with 500 38C13 tumor cells and tumor growth was observed three times per week. *Asterisks* indicate significant difference ($P < 0.05$) between the DC–CD40L group and all other groups

costimulatory molecules such as CD80 and CD86 may not be an adequate indicator to define the activation state of DC, and their expression may not be sufficient for DC to fully stimulate antigen-specific T cells. Other parameters such as cytokine production may help define the activation state of DC. We have shown that Id-CD40L fusion protein is capable of activating the bone marrow-derived DC by upregulating the costimulatory molecules (Fig. 1). In addition, we found that Id-CD40L fusion protein was able to induce DC to produce IL-12 (Fig. 2). Taken together, these results demonstrate that Id-CD40L fusion protein was able to activate DC to become more potent stimulators of quiescent T cells. These in vitro observations were in accordance with the in vivo results. C3H mice immunized with DC loaded with Id-CD40L fusion protein generated superior anti-Id antibody responses (Fig. 3), T-cell responses (Fig. 4), and retarded tumor growth (Fig. 6) when compared to DC loaded with Id alone. Presumably, Id-CD40L fusion protein possesses the ability of targeting Id to DC through the ligation of CD40 and CD40L. Meanwhile, the interaction of CD40 and CD40L also transmits signals to activate DC and facilitates the processing of the Id to be presented to T cells with much greater efficiency.

In the tumor-bearing host, the immune system may have already been exposed to the tumor antigens, and thus may already have become "tolerant" to them. In order to break this tolerance, one way is to employ the potent activated APC derived from mature DC. It has been suggested that immature DC may elicit anergy or tolerance, whereas mature DC will induce productive immunity [2, 14, 31, 38]. The CD40–CD40L combination can act as a 'switch' to convert the unresponsive T cell to become responsive [11, 39]. Reports from other laboratories have shown that ligation of CD40 on DC increases the capacity of DC to stimulate

T-cell proliferation and IFN-γ production [7]. These observations suggest that immunization with DC armed with the Id-CD40L fusion protein may help break the "Id tolerance". We found that mice immunized with DC armed with Id-CD40L were indeed capable of inducing Id-specific T cell proliferation (Fig. 4) and IFN-γ (Fig. 5A) production.

With regard to the IgG subclass profile, mice immunized with DC pulsed with Id-GM–CSF fusion protein generated substantial proportions of IgG2a anti-Id antibodies. In contrast, mice immunized with Id-GM–CSF fusion protein without DC exclusively induced the IgG1 isotype [45]. Our findings are consistent with these studies. Mice immunized with DC pulsed with the Id-CD40L fusion protein induced high levels of IgG2a anti-Id antibodies (Fig. 3B), and IFN-γ production (Fig. 5A). Taken together, these results suggest that immunization with DC pulsed with Id-CD40L fusion proteins induces anti-Id immune responses which include a Th1 response. The role of T cells in conferring in vivo anti-tumor immunity in mice immunized with Id is not clear [28, 45]. In the present study, we failed to identify the presence of anti-Id CTL toward 38C13 tumor cells (data not shown). Thus, these results suggest that the induction of humoral immunity seems to be indispensable for inducing the therapeutic effect in 38C13 lymphoma. Nevertheless, although T cells may not play a direct role as effectors in the inhibition of the growth of 38C13 lymphoma, Th cells may play an important role here. In addition to promoting the antibody response, IFN-γ has been shown to recruit host cells to induce tumor rejection [20]. These effects appear to be achieved by the vaccination of DC pulsed with Id-CD40L. Therefore, our findings provide the rationale for the use of CD40L linking to Id for DC pulsing in lymphoma clinical trials, and suggest a new strategy with possible implications for DC immunization against other tumors.

Many current studies are utilizing DC in an attempt to confer immunity against tumor antigens. Optimizing DC activation by stimulation with multiple agents may improve these efforts towards the generation of a potent antitumor response in vivo. In the present study, we have demonstrated that Id-CD40L fusion protein can augment the stimulatory capacity of DC. Although their in vivo antitumor effect is not sufficient to overcome the tumorigenicity of the very aggressive B lymphoma 38C13, their therapeutic effect should be improved by combining them with other treatments, for example, cytokine adjuvant therapy and/or chemotherapy. Studies in this regard are currently under investigation in our laboratory. This is a starting point that may help to design strategies leading to more effective treatment.

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