The Journal *of* Immunology

This information is current as of November 22, 2019.

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Targeting with Bovine CD154 Enhances Humoral Immune Responses Induced by a DNA Vaccine in Sheep[1]

Sharmila Manoj,*† Philip J. Griebel,* Lorne A. Babiuk,* and Sylvia van Drunen Littel-van den Hurk[2]*

CD40-CD154 interactions play an important role in regulating humoral and cell-mediated immune responses. Recently, these interactions have been exploited for the development of therapeutic and preventive treatments. The objective of this study was to test the ability of bovine CD154 to target a plasmid-encoded Ag to CD40-expressing APCs. To achieve this, a plasmid coding for bovine CD154 fused to a truncated secreted form of bovine herpesvirus 1 glycoprotein D (tgD), pSLIAtgD-CD154, was constructed. The chimeric tgD-CD154 was expressed in vitro in COS-7 cells and reacted with both glycoprotein D- and CD154-specific Abs. Both tgD and tgD-CD154 were capable of binding to epithelial cells, whereas only tgD-CD154 bound to B cells. Furthermore, dual-labeling of ovine PBMCs revealed that tgD-CD154 was bound by primarily B cells. The functional integrity of the tgD-CD154 chimera was confirmed by the induction of both IL-4-dependent B cell proliferation and tgD-specific lymphoproliferative responses in vitro. Finally, sheep immunized with pSLIAtgD-CD154 developed a more rapid primary tgD-specific Ab response and a significantly stronger tgD-specific secondary response when compared with animals immunized with pSLIAtgD and control animals. Similarly, virus-neutralizing Ab titers were significantly higher after secondary immunization with pSLIAtgD-CD154. These results demonstrate that using CD154 to target plasmid-expressed Ag can significantly enhance immune responses induced by a DNA vaccine. *The Journal of Immunology,* **2003, 170: 989–996.**

The use of Ag-encoding DNA to stimulate long-lasting cell-mediated and humoral immune responses offers several advantages in terms of economy, adaptability, and, most of all, simplicity of vaccine production. Numerous studies have confirmed that plasmid-expressed Ag can induce protective immune responses in mice, but similar vaccine efficacy has not been observed for large-animal species (1). Although we have developed several strategies to optimize the efficacy of DNA vaccines against bovine herpesvirus 1 (BHV-1)[3] (1), further improvements in immunogenicity are desirable.

The induction of immune responses by DNA vaccines in large animals has been limited by a number of factors, including low transfection efficiency, which results in inadequate levels of Ag expression. Strategies are being developed to enhance the immunogenicity of DNA vaccines by effectively targeting the limited amount of Ag expressed to APCs. Ags linked to molecules such as CTLA-4 and L-selectin have been targeted to APCs, enhancing immune responses in different species with varying degrees of success (2–4). Recently, CD154 trimer linked to carcinoembryonic Ag induced effective tumor-protective immunity by activating both naive T cells and dendritic cells (DCs) in carcinoembryonic Ag-transgenic mice (5).

Both CD154 and the CD40R belong to the TNF superfamily (6). CD154 (CD40 ligand, TNF-related activation protein, or gp39), a 39-kDa glycoprotein, is expressed as a type II integral membrane protein on the surface of activated T cells, basophils, and mast cells. Its receptor, CD40, is a 45- to 50-kDa surface protein that is expressed on B cells, DCs, macrophages, and Langerhans cells, and also on nonhemopoietic cells including endothelial cells, fibroblasts, and epithelial cells. CD40-CD154 interactions between DCs and T cells provide signals for activation and maturation of DCs (7). CD154 ligation of CD40 on B cells influences various stages in B cell development (8–10), including secretion of cytokines (11) and Ig isotype switching. Bovine CD154 has been cloned and sequenced (12), and the kinetics and expression of CD154 on bovine T cells (13) and its role in bovine B cell development and differentiation (14, 15) have been investigated. Coadministration of a plasmid encoding CD154 and a plasmid encoding the Ag of interest enhanced humoral and cellular immune responses in mice (16–19).

In contrast to studies that used CD154 primarily as an adjuvant, our goal was to explore the dual role of bovine CD154 as both an adjuvant and a vaccine-targeting molecule. The Ag chosen for these studies was BHV-1 glycoprotein D (gD), because vaccination with gD has been shown to protect cattle from BHV-1 infection (20). A DNA vaccine encoding gD has induced effective cell-mediated immune responses, but the Ab response, in terms of kinetics and magnitude, has not been satisfactory. As a result, the gD-based DNA vaccine induced moderate disease protection, similar to that provided by killed or live viral vaccines (21).

To enhance immune responses to plasmid-expressed Ag, a plasmid encoding a truncated, secreted form of gD (tgD) fused to bovine CD154 (tgD-CD154) was constructed. We postulated that the tgD-CD154 chimera would bind CD40 on APCs and enhance Ag processing and presentation. In addition, if tgD-CD154 formed soluble dimers or trimers, then the chimeric protein might induce APC activation with increased expression of cosignals. Our results indicate that the in vitro-expressed tgD-CD154 chimera retained the conformation and function of both tgD and CD154. Furthermore,

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[3] Abbreviations used in this paper: BHV-1, bovine herpesvirus 1; gD, glycoprotein D; tgD, truncated gD; DC, dendritic cell; RT, room temperature; SI, stimulation index.

immunization with plasmid encoding tgD-CD154 elicited significantly enhanced tgD-specific Ab titers in comparison to immunization with plasmid encoding tgD. These results provide promising evidence that fusion of vaccine Ags with CD154 can enhance humoral immune responses induced by DNA immunization.

Materials and Methods

All restriction enzymes were purchased from Amersham Pharmacia (Baie d'Urfe, Quebec, Canada). All cell culture incubations were performed at 37°C in a humidified 5% CO_2 atmosphere unless otherwise specified.

Generation of DNA constructs

The construction of pSLIAtgD has been described previously (22). pc-bCD40L encoding bovine CD154 was a generous gift from Dr. M. Estes (University of Missouri, Columbia, MO). To create pSLIAtgD-CD154 (Fig. 1), a 645-bp segment (aa 46–261) of the bovine CD154 coding sequence was amplified by PCR from pc-bCD40L using primers 5′-AT TACGCGTCTTCACAGACGATTGGAC-3′ and 3′-CGGGCTAGCTGCT TTCCTGGATTGTGA-5′, and inserted in frame with the coding sequence of tgD into the EcoRV site on pSLIAtgD. Plasmids were grown in Escherichia coli DH5α and purified by anion exchange resin (Qiagen, Mississauga, Ontario, Canada), followed by Triton X-114 (Sigma-Aldrich, Oakville, Ontario, Canada) extraction (23). Endotoxin levels in DNA stocks were verified to <0.10 endotoxin U/mg DNA (<10 pg/mg DNA), using the Limulus amebocyte lysate QLC-1000 kit (BioWhittaker, Walkersville, MD). The concentration was assessed spectrophotometrically, and constructs were confirmed by restriction enzyme digestion and agarose gel electrophoresis.

Transfection and production of recombinant proteins

To obtain tgD and tgD-CD154 for in vitro assays, COS-7 cells were cultured at a concentration of 2.5×10^5 cells/well in 6-well plates (Corning, Corning, NY) in DMEM (Canadian Life Technologies, Burlington, Ontario, Canada) with 10% FBS (Canadian Life Technologies). Cells were transfected with pSLIAtgD or pSLIAtgD-CD154 using Lipofectamine reagent (Canadian Life Technologies) according to the manufacturer's instructions and cultured with Optimem for 36 h before collecting supernatants. These supernatants were concentrated 10-fold using Biomax filters (Millipore, Bedford, MA) and analyzed by Western blotting to determine that both proteins were present in equivalent quantities.

Radioimmunoprecipitation

COS-7 cells were transfected with pSLIAtgD or pSLIAtgD-CD154. After incubation for 24 h, the transfected cells were incubated with fresh methionine- and cysteine-free MEM (Sigma-Aldrich) for 2 h. Subsequently,
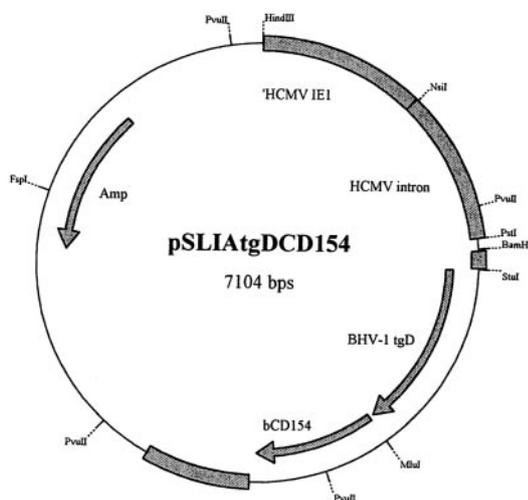
[35S]methionine and cysteine (Mandel Scientific, Guelph, Ontario, Canada) were added, and the cells were incubated overnight at 37°C. The culture supernatants were collected and incubated with a 1/100 dilution of mouse ascites containing gD-specific mAbs (clones 10C2, 3D9S, 4C1, 2C8, and 9D6; Ref. 24) for 4 h on ice. Protein A-Sepharose beads (Amersham Pharmacia) were coated with rabbit anti-mouse IgG (Cappel, Aurora, OH) and subsequently incubated overnight at 4°C with the protein-bound gD-specific mAbs. The beads were washed with 0.01 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, and 1 mM PMSF, and resuspended in electrophoresis sample buffer for analysis by SDS-PAGE and autoradiography.

Production of bovine CD154-specific rabbit serum

To create pGST-CD154, a 782-bp fragment from pc-bCD40L encoding full-length bovine CD154 was amplified by PCR using primers 5′-CG GAATTCCTTCACAGACGATTGGAC-3′ with an EcoRV site and 5′-CCGCTCGAGTGCTTTCCTGGATTGTGA-3′ with an XhoI site. The amplified DNA fragment was inserted in frame with the coding sequence of GST between EcoRV-XhoI sites in the pGEX-5 vector (Invitrogen, Burlington, Ontario, Canada). GST-CD154 was produced as an insoluble protein in lysates of DH5α cells and separated on a 10% polyacrylamide gel. The gel was copper stained (25) to identify GST-CD154, which was subsequently excised, homogenized, and mixed with VSA3 adjuvant (26). Rabbits were immunized twice with the GST-CD154 formulation. Two weeks after the secondary immunization, the rabbits were bled, and they were sacrificed 10 days later.

Western blot

COS-7 cell supernatants containing tgD or tgD-CD154 were separated on 10% polyacrylamide gels under reducing or nonreducing conditions (27), and the proteins were transferred to nitrocellulose membrane (Millipore). The membrane was washed in TBST (0.15 M Tris, 0.02 M NaCl (pH 7.5), and 0.1% Tween 20), and incubated overnight with TBST containing 5% skim milk powder. Subsequently, the membrane was incubated with gD-specific mAb mixture (1:5000) or bovine CD154-specific polyclonal rabbit serum (1:1000) for 1 h at room temperature (RT). This was followed by incubation with alkaline phosphatase-conjugated mouse- or rabbit-specific IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a dilution of 1/5000 for 1 h at RT. The bound Abs were visualized with 10 ml of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma-Aldrich) substrate.

Flow cytometry

mAbs specific for sheep CD4 (clone 17D-13; IgG1) and γδ TCR (clone 86D; IgG1) on T cells, or CD72 on B cells (clone DU2-104.4; IgM) were produced from hybridomas generously provided by Dr. W. Hein (Ag Research, Wallaceville, New Zealand). mAbs specific for monocytes (clone DH59B; IgG1), sheep IgM (clone PIg45A; IgG2b), and sheep IgG1 (clone Big715A; IgG1) were purchased from VMRD (Pullman, WA). FITC-conjugated goat IgG specific for murine IgG1 and IgM and PE-conjugated goat IgG specific for murine IgG2a and IgG2b were purchased from Southern Biotechnology (Birmingham, AL). gD-specific mAbs were obtained as described earlier (24). All incubations were performed for 30 min on ice.

Single labeling of Madin-Darby bovine kidney (MDBK) cells and ovine B cells and dual labeling of ovine PBMCs were performed by incubating the cells with medium or COS-7 cell supernatants containing tgD or tgD-CD154. The cells were washed in PBS before incubation with gD-specific mAb mixture for single labeling, or both gD-specific mAb 1G6 (IgG2a) and mAbs to various cell surface molecules for dual labeling. After three washes, appropriate isotype-specific, FITC- and PE-conjugated goat anti-mouse IgG (Southern Biotechnology) were added, and the cells were further incubated. After a final wash, the cells were fixed with 2% paraformaldehyde until analyzed. All samples were analyzed with a FACScan (BD Biosciences, Mountain View, CA) flow cytometer, and the CellQuest program (BD Biosciences) was used for data acquisition and analysis. Nonspecific mAb binding was quantified with isotype-matched, irrelevant mAbs (Sigma-Aldrich), and 5000 events were analyzed for each sample.

Competition binding assay

Ovine B cells, clone 2, were suspended at 5×10^4/well and incubated for 10 min with 2-fold serial dilutions of human CD154 ligand trimer (R&D Systems, Minneapolis, MN) starting at a final concentration of 20 μg/ml. COS-7 cell culture supernatants containing tgD-CD154 were then added to the cells and incubated further for 20 min. The cells were washed in PBS before incubation with gD-specific mAb (1G6). After three washes, FITC-conjugated goat anti-mouse IgG (Southern Biotechnology) was added, and the cells were further incubated. After a final wash, the cells were fixed

FIGURE 1. Plasmid pSLIAtgD-CD154. A 645-bp segment (aa 46–261) of bovine CD154 was cloned into the EcoRV site in frame with the coding sequences of tgD in the expression vector pSLIA0, containing the human cytomegalovirus immediate early gene promoter and bovine growth hormone poly(A) tail.

with 2% paraformaldehyde until analyzed. All samples were analyzed with a FACScan (BD Biosciences) flow cytometer, and the CellQuest program (BD Biosciences) was used for data acquisition and analysis. Nonspecific mAb binding was quantified with isotype-matched, irrelevant mAbs (Sigma-Aldrich), and 5000 events were analyzed for each sample.

IL-4 bioassay

An IL-4 bioassay was performed with cloned ovine B cells, clone 2 (28), which are dependent on CD154 costimulation with γ-chain-common cytokines for sustained growth. Fifty microliters containing 8×10^5 cells/ml of clone 2 B cells and COS-7 cell supernatants containing tgD or tgD-CD154, or gamma-irradiated J558L cells expressing murine CD154 (29), were incubated for 72 h with 100 μl of medium or recombinant human IL-4 at 5 ng/ml (PeproTech, London, U.K.) in a U-bottom microtiter plate (Nalge Nunc International, Rochester, NY). [*methyl*-³H]Thymidine (Amersham Pharmacia) was added at 0.4 μCi/well during the last 8 h of culture before harvesting. Incorporation of [³H]thymidine was measured using a liquid scintillation counter (Beckman 1701; Beckman Instruments, Fullerton, CA).

Lymphocyte proliferation assay

Blood was collected into EDTA-treated vacutainers (BD Biosciences), and PBMCs were isolated as previously described (30) and resuspended in AIM V (Life Technologies, Grand Island, NY) with 2% FBS (Canadian Life Technologies) and 5×10^{-5} mM 2-ME (Sigma-Aldrich). To assay tgD-specific proliferative responses, 1×10^7 PBMCs isolated from naive (tgD⁻) or tgD-immunized (tgD⁺) animals were incubated for 30 min on ice with medium or COS-7 cell supernatants containing tgD or tgD-CD154, and washed to remove unbound protein. The cells were resuspended in AIM-V and 2×10^5 cells/well were cultured in triplicate wells in U-bottom microtiter plates (Nalge Nunc International) for 72 h. In addition, triplicate cultures of PBMCs from tgD⁻ or tgD⁺ animals were incubated with 1 μg/ml purified tgD for 72 h. [*methyl*-³H]Thymidine (Amersham Pharmacia) was added at 0.4 μCi/well during the last 8 h of culture. Incorporation of [³H]thymidine was measured using a liquid scintillation counter (Beckman 1701; Beckman Instruments). Proliferative responses were expressed as a stimulation index (SI), where SI represents cpm in the presence of Ag divided by cpm in the absence of Ag.

DNA immunization of sheep

Twenty-four male and female, 6-mo-old Suffolk sheep (Department of Poultry and Animal Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada) were randomly assigned to four experimental groups: group I, pSLIAtgD ($n = 7$); group II, pSLIAtgD-CD154 ($n = 7$); group III, pSLIA0 ($n = 5$); and group IV, saline ($n = 5$). Sheep were injected intradermally in the ear with five separate injections of 100 μg plasmid to deliver a total dose of 500 μg of plasmid per animal. Secondary DNA immunizations were performed in the same manner 9 wk later. Sera were collected at weekly intervals to assay tgD-specific Ab titers. The experiment was conducted in accordance with the guidelines provided by the Canadian Council on Animal Care.

ELISA

Polystyrene microtiter plates (Immulon II; Dynatech Laboratories, Alexandria, VA) were coated with 0.05 μg/well tgD (20) overnight at 4°C. Plates were washed in PBS (0.137 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, and 0.001 M Na₂H₂PO4 (pH 7.4)) with 0.05% Tween 20 (PBST) before the addition of 4-fold dilutions of sheep sera prepared in PBST. After a 2-h incubation at RT, plates were washed in PBST and affinity-purified alkaline phosphatase-conjugated mouse anti-sheep IgG (Kirkegaard and Perry Laboratories) was added at a dilution of 1/5000. After another hour at RT, plates were washed, and the reactions were visualized with 0.01 M *p*-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was read on a model 3550 microplate reader (Bio-Rad Laboratories, Randolph, MA) at 405 nm, with a reference wavelength of 490 nm. The tgD-specific Ab titers were calculated based on the cutoff value set at an OD reading corresponding to the reciprocal dilution of the standard positive control serum at >10,240.

Virus neutralization assay

Virus neutralization assays were performed as described previously (20). MDBK cells were cultured overnight in 96-well tissue culture plates (Falcon; BD Biosciences). Sera from sheep were serially diluted 2-fold and known BHV-1 high-positive, low-positive, and negative sera were used as controls. The Cooper strain of BHV-1 was diluted 1/1 with 100 μl of each serum sample and allowed to incubate at 37°C for 1 h. The serum-virus mixture was then added to duplicate MDBK cell cultures and incubated for

48 h. The plates were stained with crystal violet to visualize viral plaques before counting, and virus neutralization titers were expressed as the reciprocal of the highest dilution of serum that caused a 50% reduction in plaques relative to the virus control.

Statistical analyses

All data were analyzed with the aid of a statistical software program (Systat 10.0; SPSS, Chicago, IL). Ab titers were transformed before performance of the analysis by log transformation, because they were not normally distributed. Differences in total IgG titer between the groups across various weeks were examined by repeated measures ANOVA. The Mann-Whitney *U* test was used to compare the virus neutralization titers between the groups.

Results

In vitro-expressed tgD-CD154 is recognized by gD- and bovine CD154-specific Abs

To determine whether tgD-CD154 could be expressed as a chimeric protein in eukaryotic cells, COS-7 cells were transfected with pSLIAtgD or pSLIAtgD-CD154 (Fig. 1). Proteins in the supernatants from the transfected cells were precipitated with a mixture of gD-specific mAbs (24) (Fig. 2*a*, *lanes 1* and *2*). As expected, a 61-kDa protein (24) was precipitated from the supernatant of pSLIAtgD transfected cells. In the supernatants of
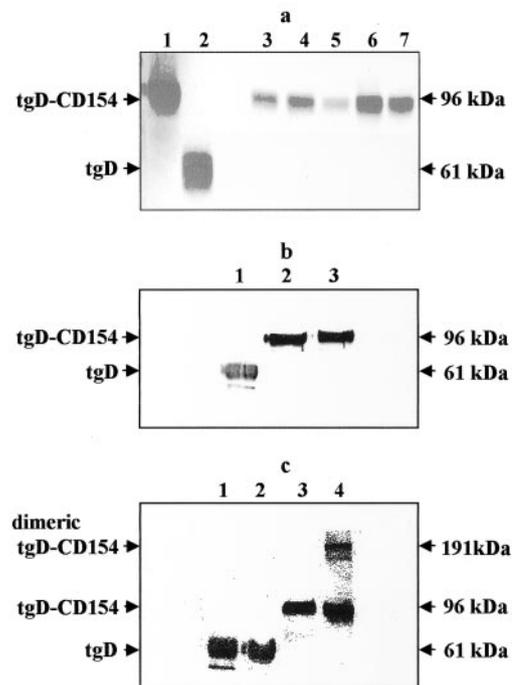

FIGURE 2. In vitro expression of tgD and tgD-CD154. *a*, COS-7 cells were transiently transfected with pSLIAtgD (*lane 2*) or pSLIAtgD-CD154 (*lanes 1*, *3*, *4*, *5*, *6*, and *7*). Radiolabeled proteins in culture supernatants were precipitated with gD-specific mAbs and separated on a 8.5% reducing gel. *Lanes 1* and *2*, Proteins precipitated by a gD-specific mAb mixture from supernatants of pSLIAtgD-CD154 and pSLIAtgD transfected cells, respectively. *Lanes 3*, *4*, *5*, *6*, and *7*, Proteins precipitated from supernatants of pSLIAtgD-CD154 transfected cells by gD-specific mAbs 10C2, 3D9S, 9D6, 2C8, and 4C1, respectively. *b*, Western blot of culture supernatants from pSLIAtgD (*lane 1*) or pSLIAtgD-CD154 (*lanes 2* and *3*) transfected COS-7 cells following separation on a 10% reducing gel. The blots were incubated with gD-specific mAb mixture (*lanes 1* and *2*) or CD154-specific rabbit serum (*lane 3*). *c*, Western blot of culture supernatants from pSLIAtgD (*lanes 1* and *2*) or pSLIAtgD-CD154 (*lanes 3* and *4*) transfected COS-7 cells, separated on a 10% gel under reducing (*lanes 1* and *3*) or nonreducing (*lanes 2* and *4*) conditions. The blots were incubated with a gD-specific mAb mixture. The positions of tgD (61 kDa), tgD-CD154 (96 kDa), and dimeric tgD-CD154 (191 kDa) are indicated in the margins.

the pSLIAtgD-CD154 transfected cells, a 96-kDa protein was detected, which corresponds to the combined molecular mass of tgD (61 kDa) and CD154 (35 kDa). To investigate the conformation of tgD within the chimeric protein, pSLIAtgD-CD154 transfected COS-7 cell supernatants were precipitated with five different mAbs (clones 10C2, 3D9S, 9D6, 2C8, and 4C1) directed toward gD epitopes IIIa, IV, Ib, IIIc, and IIIb, respectively (24). All mAbs, except 3D9S, recognize conformation-dependent epitopes on gD. mAbs specific for the various gD epitopes (24) also reacted with tgD-CD154, suggesting that the conformation of tgD was conserved within the chimeric protein (Fig. 2a). Bovine CD154-specific mAbs were not available, so a CD154-specific rabbit serum was raised. Western blots with tgD or tgD-CD154 in transfected cell supernatants were incubated with the gD-specific mAb mixture or with the CD154-specific rabbit serum. As illustrated in Fig. 2b, the gD-specific mAb mixture again reacted with 61-kDa (*lane 1*) and 96-kDa (*lane 2*) proteins, which correspond to the apparent molecular mass of tgD and tgD-CD154, respectively, whereas the bovine CD154-specific rabbit serum reacted with only a 96-kDa protein (*lane 3*) in the supernatants of cells transfected with pSLIAtgD-CD154. These results confirmed the expression of CD154 in the chimeric protein.

A dimeric form of in vitro-expressed tgD-CD154 is recognized by gD-specific Abs

rCD154 can form soluble monomers, dimers, or trimers, and each form displays different abilities to deliver biological signals (31). To determine whether dimeric or trimeric forms of tgD-CD154 could be detected, transfected COS-7 cell supernatants containing tgD or tgD-CD154 were separated by electrophoresis under reducing or nonreducing conditions and visualized by Western blotting. Under reducing conditions, both tgD and tgD-CD154 (Fig. 2c, *lanes 1* and *3*) were recognized in transfected cell supernatants as a single band, representing monomeric forms with apparent molecular masses of 61 kDa and 96 kDa, respectively. Under nonreducing conditions, tgD was again detected as a 61-kDa monomer (Fig. 2c, *lane 2*). The tgD-CD154 monomer (Fig. 2c, *lane 4*) migrated slightly faster and appeared to have a molecular mass of 86 kDa, which is likely due to the presence of intramolecular disulfide bonds within tgD-CD154. An additional band, with an apparent molecular mass of 191 kDa, was observed in supernatants of pSLIAtgD-CD154 transfected cells. This high-molecular mass band was interpreted as a dimeric form of the tgD-CD154 molecule because a CD154-Fc fusion construct was observed to form disulfide-linked homodimers under nonreducing conditions (31). The present results suggest that, due to the intrinsic ability of CD154 to dimerize, at least a portion of tgD-CD154 formed dimers with an apparent molecular mass of 191 kDa.

In vitro-expressed tgD-CD154 binds to bovine epithelial cells and ovine B cells

A putative receptor on bovine epithelial cells has been identified for BHV-1 gD (32) and CD154 binds to CD40 expressed on B lymphocytes (33). To further evaluate whether the tgD-CD154 chimera expressed in transfected cells was conformationally correct, we investigated the ability of each protein to bind their respective receptors. MDBK cells and clone 2 B cells were incubated with pSLIAtgD or pSLIAtgD-CD154 transfected COS-7 cell culture supernatants. Ovine cells were used to test the tgD-CD154 interaction with CD40 because of the availability of a cloned B cell line (29) and the 95% homology between ovine and bovine CD40 (gene bank identifiers 18447758 and 1480642, respectively). Both tgD and tgD-CD154 were bound by MDBK cells (Fig. 3a), but only tgD-CD154 was bound by clone 2 B cells (Fig. 3b). To further confirm that this binding was due to inter-
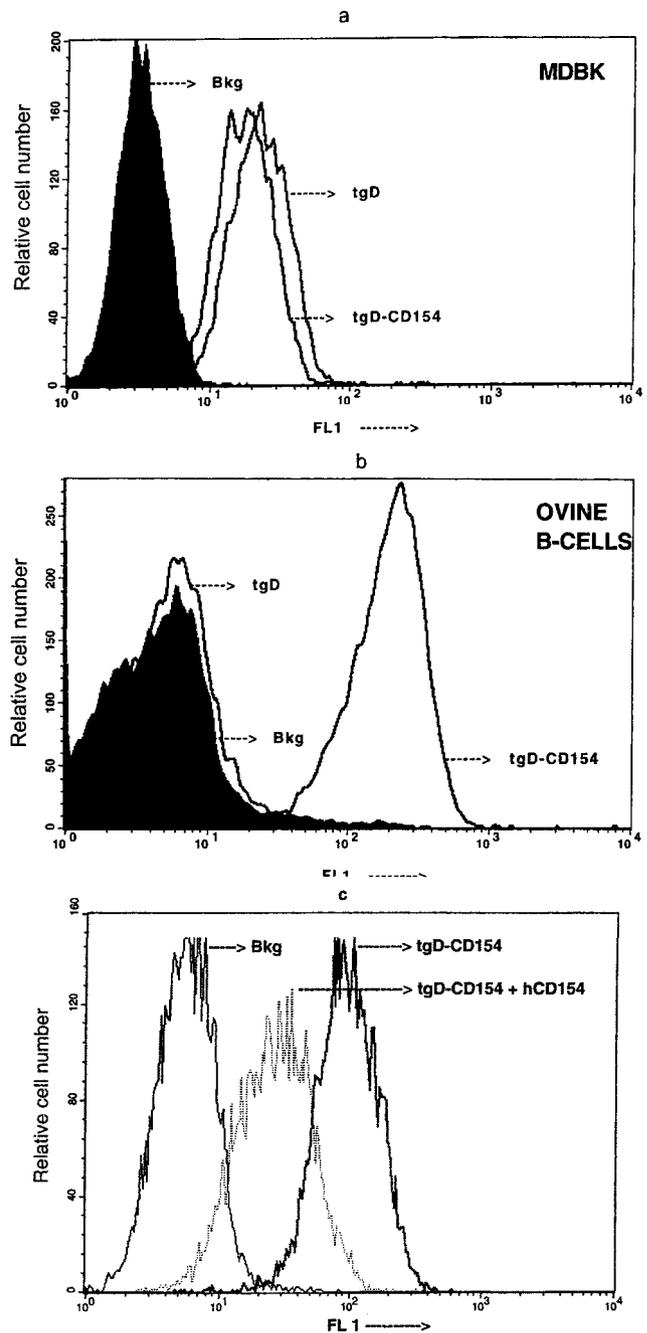

FIGURE 3. Binding of tgD and tgD-CD154 to bovine epithelial cells (MDBK) and ovine B cells (clone 2). Cell labeling was performed by incubating supernatants from pSLIAtgD or pSLIAtgD-CD154 transfected COS-7 cells with MDBK cells (*a*) and ovine B cells (*b*), and ovine B cells preincubated with recombinant human CD154 trimer (*c*). Bound protein was detected with a gD-specific mAb mixture, followed by FITC-conjugated goat anti-mouse Ig. Background labeling (Bkg) shows labeling of cells by medium alone.

action between CD40 and bovine CD154, a competitive binding assay was performed using various concentrations of human CD154 ligand trimer as a competitor. As shown in Fig. 3c, the binding of tgD-CD154 in transfected COS-7 cell supernatants to clone 2 B cells was reduced by 50% at the highest concentration of human CD154 ligand trimer tested.

To determine whether tgD-CD154 expressed by transfected cells could target B cells that had not undergone prior in vitro manipulation, dual labeling of PBMCs was used to identify which

cell types bound tgD and tgD-CD154. As illustrated in Fig. 4, 1.88% and 14.4% of the cells binding tgD and tgD-CD154, respectively, also expressed CD72, a pan B cell marker (28). In contrast, there was no difference between the level of tgD and tgD-CD154 bound by CD4$^+$ T cells or DH59B$^+$ monocytes (data not shown). These results confirmed that, within the tgD-CD154 chimera, tgD retained the capacity to bind to bovine epithelial cells, and CD154 retained the capacity to bind to CD40 on B cells, both cultured and freshly isolated. Furthermore, these observations indicated that CD154 could specifically target tgD to B cells.

In vitro-expressed tgD-CD154 induces IL-4-dependent proliferation of ovine B cells

One of the consequences of CD40-CD154 interaction in T cell-dependent B cell activation is the induction of IL-4-dependent B cell proliferation (34). It has been shown that with the exception of monomeric CD154, most other forms of CD154, such as dimeric, trimeric, and membrane-bound CD154, can induce IL-4 responsiveness in B cells (34). To determine whether the chimeric tgD-CD154 induced CD40 signaling, ovine B cells (29) were cultured with pSLIAtgD or pSLIAtgD-CD154 transfected COS-7 cell supernatants in the presence or absence of recombinant human IL-4. Ovine B cells were also cultured with murine CD154 transfected J558L cells (29) as a positive control for IL-4 responsiveness. As shown in Fig. 5, only transfected J558L cells and pSLIAtgD-CD154 transfected COS-7 cell supernatants induced IL-4-dependent proliferation of ovine B cells. This observation confirmed that tgD-CD154 specifically bound CD40 and also induced CD40 signaling.

In vitro-expressed tgD-CD154 induces tgD-specific lymphocyte proliferative responses

From previous experiments, it was evident that linking CD154 and tgD could specifically target tgD to ovine B cells. To determine whether Ag targeting to B cells induced Ag-specific lymphocyte proliferative responses in vitro, we compared the proliferative re-
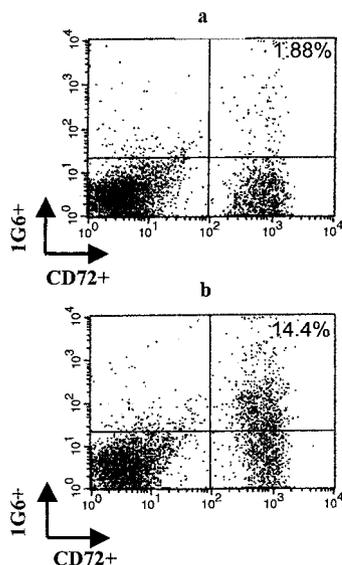

FIGURE 5. CD40 signaling induced by tgD-CD154. Triplicate cultures of clone 2 B cells (4×10^4 cells/well) were incubated with pSLIAtgD or pSLIAtgD-CD154 transfected COS-7 cell supernatants. Cultures were incubated for 72 h with either 100 μl/well of 5 ng/ml recombinant human IL-4 (+IL-4) or medium (−IL-4). As a positive control, gamma-irradiated J558L-CD154 cells (4×10^3 cells/well) were added to triplicate cultures of clone 2 B cells. Cell proliferation was detected by adding [*methyl-*^{3}H]thymidine during the last 8 h of culture. Data are shown as average cpm ± 1 SD for triplicate cultures.

sponses of PBMCs isolated from naive (tgD$^-$) or tgD-immunized (tgD$^+$) sheep. As shown in Fig. 6, PBMCs from tgD$^+$ sheep specifically responded to tgD-CD154 transfected cell supernatants. Interestingly, PBMCs from tgD$^+$ sheep displayed comparable proliferative responses, regardless of whether they were pulsed with pSLIAtgD-CD154 transfected COS-7 cell supernatants or incubated for 72 h with 1 μg/ml purified tgD. Furthermore, pSLIAtgD-CD154 transfected cell supernatants did not induce nonspecific lymphoproliferative responses because there was not an increased proliferative response by PBMCs isolated from tgD$^-$ sheep. Collectively, these observations indicate that using a tgD-CD154 chimera to target B cells can result in the processing and presentation of tgD.

Immunization with pSLIAtgD-CD154 induces significantly higher tgD-specific Ab titers than immunization with pSLIAtgD

The ability of pSLIAtgD or pSLIAtgD-CD154 to induce tgD-specific Ab responses in vivo was compared in sheep. Animals were immunized twice intradermally with 500 μg of pSLIAtgD or pSLIAtgD-CD154, and serum Ab titers were measured at weekly

FIGURE 4. Binding of tgD and tgD-CD154 to ovine PBMCs. Dual labeling was performed by first incubating PBMCs with culture supernatants of COS-7 cells transfected with pSLIAtgD (*a*) or pSLIAtgD-CD154 (*b*). Cells were then incubated with a mixture of gD-specific mAb 1G6 and mAbs specific for CD72. Isotype-specific goat anti-mouse Ig conjugated to either FITC or PE was used to detect anti-gD and anti-leukocyte mAbs. Numbers inside quadrants represent the percentages of dual-labeled cells.
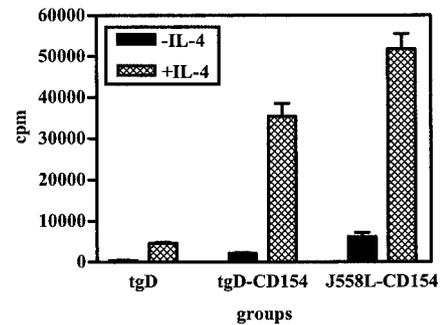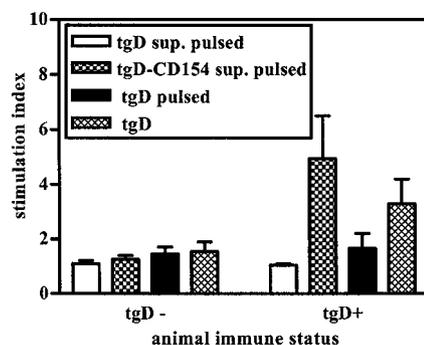

FIGURE 6. Ag-specific lymphocyte proliferation induced by tgD-CD154. PBMCs were isolated from naive (tgD$^-$) and tgD-immunized (tgD$^+$) sheep and incubated for 30 min at 4°C with either pSLIAtgD (tgD sup. pulsed) or pSLIAtgD-CD154 (tgD-CD154 sup. pulsed) transfected COS-7 cell supernatants or with 1 μg/well of purified tgD (tgD pulsed). Subsequently, PBMCs were washed twice, and 2×10^5 cells/well were cultured for 72 h. As a positive control, 2×10^5 PBMCs/well were also cultured for 72 h with 1 μg of purified tgD (tgD). Cell proliferation was detected by adding [*methyl-*^{3}H]thymidine during the last 8 h of culture. Data are shown as mean SI (cpm of Ag-pulsed cells/cpm of control cells) ± 1 SEM of triplicate cultures.

intervals. As there were no statistically significant differences between the control groups immunized with either pSLIA0 or saline by repeated measures ANOVA, these two groups were combined when presenting data. At 2 wk after primary immunization, the pSLIAtgD-CD154-immunized group had significantly higher tgD-specific Ab titers relative to control groups ($p < 0.05$), whereas the titers of the pSLIAtgD-immunized and control groups were not significantly different until 3 wk after primary immunization ($p < 0.05$) (Fig. 7a). Furthermore, the pSLIAtgD-CD154-immunized group had significantly higher serum Ab titers than both the control groups at 2, 3, 4, 6, and 8 wk post-secondary immunization ($p < 0.01$), and the pSLIAtgD-immunized group at 3, 4, and 6 wk post-secondary immunization ($p < 0.05$). The serum Ab titers of animals immunized with pSLIAtgD were significantly different from the titers of the control groups at 2, 3, 4, and 6 wk post-secondary immunization ($p < 0.05$). These results indicate that immunization with pSLIAtgD-CD154 induced both a more rapid

FIGURE 7. Serum Ab response induced by immunization with pSLIAtgD or pSLIAtgD-CD154. Suffolk sheep were injected intradermally with 500 μg of pSLIAtgD (■, $n = 7$) or pSLIAtgD-CD154 ($\triangle$, $n = 7$) or with either pSLIA0 or saline (○, $n = 10$). a, Geometric mean serum ELISA titers $\pm$ SEM following primary and secondary immunization (arrows). The gD-specific Ab titers were calculated based on the cutoff value set at an OD reading corresponding to the reciprocal dilution of the standard positive control serum at $\geq 10,240$. b, Mean virus neutralization titers $\pm$ SEM at 4 wk after primary and 3 wk after secondary immunization. The virus neutralization titers are expressed as the reciprocal of the highest dilution of serum that caused a 50% reduction in viral plaques relative to the virus control. *, Significant differences ($p < 0.05$) between the pSLIAtgD- and pSLIAtgD-CD154-immunized groups are indicated.

primary Ab response and a significantly enhanced secondary Ab response when compared with immunization with pSLIAtgD. In contrast, the average tgD-specific lymphocyte proliferative responses of PBMCs from the pSLIAtgD- (SI ranging from 5.3 to 50) and pSLIAtgD-CD154- (SI ranging from 2.1 to 26.3) immunized groups tended to be higher in comparison to the control groups (SI ranging from 1.8 to 7.2), but there was no difference between the pSLIAtgD-CD154- and pSLIAtgD-immunized groups (data not shown).

To assess the biological activity of Abs induced by immunization with pSLIAtgD and pSLIAtgD-CD154, virus neutralization titers were assayed for sera collected 4 wk after primary and 3 wk after secondary immunization. Fig. 7b shows that at 4 wk after primary immunization, there was no significant difference between the virus neutralization titers of animals immunized with pSLIAtgD or pSLIAtgD-CD154. However, at 3 wk after secondary immunization, animals immunized with pSLIAtgD-CD154 had significantly higher virus-neutralizing Ab titers than animals immunized with pSLIAtgD ($p < 0.05$).
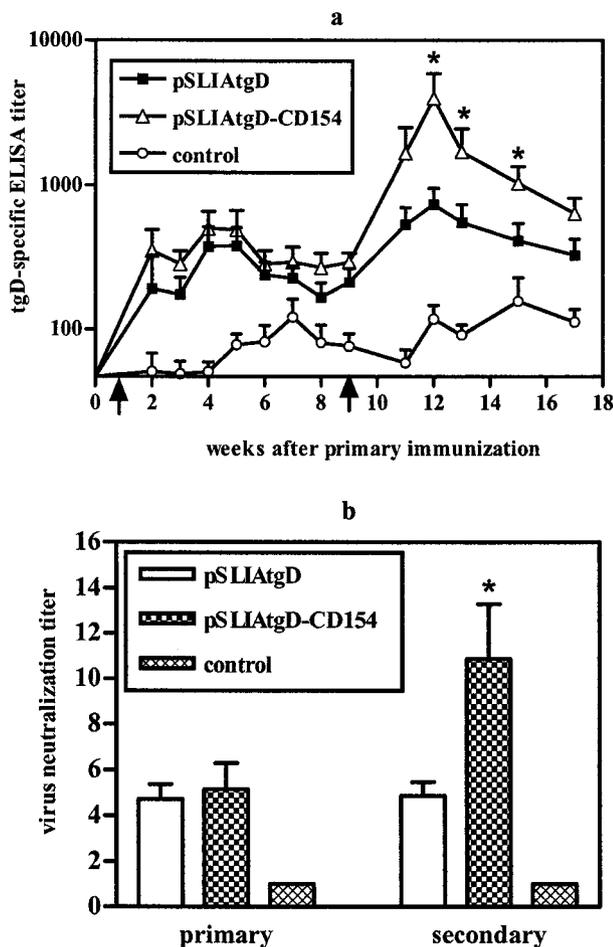
Discussion

Studies on the interactions between CD40 and CD154 have contributed extensively to our understanding of how both humoral and cellular immune responses are regulated. This knowledge has facilitated the design of approaches to modulate CD40-CD154 interactions as possible therapy for a variety of disease conditions (35–37). In addition, recent research has shown that murine CD154 can function as an adjuvant for DNA vaccines by coadministering plasmids that individually express a vaccine Ag and CD154. The expression of CD154 appeared to enhance both immune responses and disease protection (16, 18, 19). The use of immunological ligands, such as CTLA-4 and L-selectin, to target Ags has also been demonstrated to enhance DNA vaccine efficacy in mouse and sheep models (2–4). Our study explores for the first time the possibility that immune responses induced by a DNA vaccine can be enhanced when using bovine CD154 both as an adjuvant and as a targeting molecule.

Currently, DNA vaccines against BHV-1 induce an Ab response of low amplitude and short duration (1). One reason for the limited immune response induced by DNA vaccines might be the inability of plasmid-encoded Ag to be effectively acquired by APC. We sought to address this potential problem by linking the coding sequences for BHV-1 tgD and bovine CD154 within the same plasmid, creating a tgD-CD154 chimera. The expression of tgD-CD154 in the supernatants of pSLIAtgD-CD154 transfected COS-7 cells was confirmed by various gD-specific mAbs, and these immunoprecipitations indicated that tgD retained its conformation within the chimeric protein. The binding of tgD-CD154 to bovine epithelial cells and ovine B cells provided further evidence that the conformation of both tgD and CD154 was retained in the chimera. Furthermore, under nonreducing conditions, a dimeric form of tgD-CD154 was identified, and the induction of IL-4 responsiveness in B cells suggested that CD154 also retained its function. Thus, these in vitro experiments indicated that both structure and function had been conserved for each protein in the tgD-CD154 chimera.

Because bovine and ovine CD40 are 95% homologous at the amino acid level, we postulated that sheep would provide a relevant animal model to evaluate the capacity of bovine CD154 to function as both an adjuvant and targeting molecule. In vitro evidence with tgD-CD154-pulsed PBMCs supported this contention (Fig. 6). Previously, immunization of sheep with tgD-expressing plasmids induced Ab responses of short duration (38). Even in our study, where pSLIAtgD-CD154-immunized sheep showed a more

rapid primary Ab response and a significantly enhanced secondary Ab response, when compared with pSLIAtgD-immunized sheep, these responses were again of short duration. Surprisingly, enhanced Ab responses post-secondary immunization in pSLIAtgD-CD154-immunized sheep did not correlate with an increase in tgD-specific T cell proliferative responses. This observation supports the conclusion that the enhanced Ab responses in the pSLIAtgD-CD154-immunized animals may not be due to increased T cell help, but that improved Ag presentation may play a direct role in modulating B cell responses.

Intradermal DNA immunization is thought to result in transfection of keratinocytes, fibroblasts, and possibly APCs such as DCs and macrophages (39, 40). Thus, tgD and tgD-CD154 expressed by transfected DCs could be presented to CD8$^+$ T cells by MHC class I. In contrast, unlike tgD, soluble tgD-CD154 secreted from transfected keratinocytes, fibroblasts, and DCs may be targeted to CD40-expressing APCs, possibly increasing the number of tgD-presenting cells by MHC class II to CD4$^+$ T cells.

Although DCs are primarily associated with activation of naive T cells, there is increasing evidence for a role in direct B cell activation (7). MacPherson et al. (41) and Wykes et al. (42) have shown that DCs can capture and store Ag in native conformation for up to 36 h in vitro and in vivo and release Ag to be recognized by B cells in secondary lymphoid tissues. Therefore, we propose the following model to explain the induction of enhanced tgD-specific Ab responses in our study (Fig. 8). The targeting of tgD-CD154 to CD40-expressing DCs may activate them, causing the up-regulation of costimulatory molecules including CD154 (43). This may result in rapid DC migration to the secondary lymphoid organs (44), where they can deliver tgD-CD154 to B cells. Due to the ability of tgD-CD154 to recognize CD40 and tgD-specific receptors on the B cells, tgD-CD154 may access tgD-specific B cells

more efficiently than tgD. Simultaneous cross-linking of CD40 and B cell receptor on resting B cells has been shown to induce a phenotype comparable to that of a germinal center B cell subpopulation (45). Activation signals for tgD-specific B cells may also be provided by tgD-CD154 in the presence of a costimulus or the activated DCs. Kikuchi et al. (46) demonstrated that DCs modified to express CD154 and pulsed with Ag directly induced Ag-specific humoral immune responses and protection from microbial challenge that was independent of Th cells. This observation is consistent with our present findings where the enhanced Ab responses occurred without an apparent increase in T cell proliferative responses. Furthermore, memory but not naive B cells can access Ag localized on DCs (47), which may be more readily available on tgD-CD154 bound to or expressed by DCs. These possibilities, together with the ability of macrophages to be recruited to the site of DNA vaccination, where they can directly activate memory T and B cells (48) during a secondary immune response, may explain the enhanced Ab titers in sheep after secondary immunization.

Our study has two significant implications with respect to targeting of vaccine Ags. First, CD154 can be used to target a plasmid-encoded vaccine Ag. The success of this strategy may be due to the fact that tgD does not bind to B cells or monocytes. Therefore, there was no competition for CD154 binding to CD40. It would be interesting to determine whether the failure of CTLA-4 to effectively target the 45W Ag in sheep was due in part to 45W Ag binding to a cellular receptor (4). Second, tgD-CD154 binding induced B cell proliferation only in the presence of a costimulus such as a cytokine. This requirement for a second signal should limit polyclonal activation of B cells, which can occur when membrane-bound CD154 is used (31). In conclusion, coexpression of CD154 can be used to enhance humoral immune responses induced by a DNA vaccine. However, when using this approach for

FIGURE 8. A model illustrates the usual route of Ag presentation (open arrows) together with possible mechanisms by which bovine CD154 may target tgD (solid arrows) and function as an adjuvant (hatched arrows).
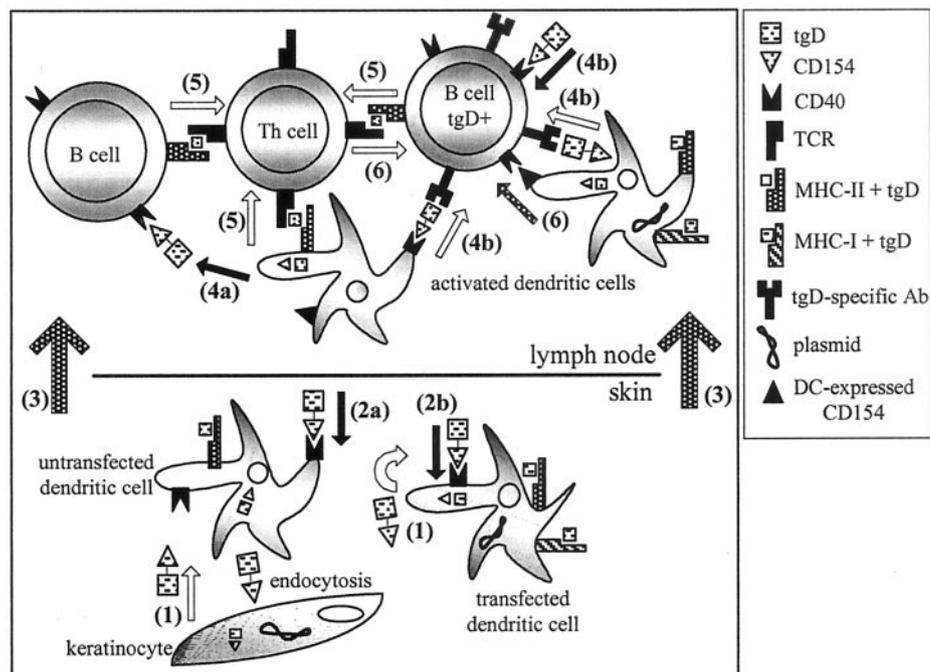
(1) plasmid transfected keratinocyte or DC secrete tgD-CD154
(2) tgD-CD154 targets and binds to (a) untransfected (b) transfected DCs possibly activating them
(3) tgD-CD154-activated DCs upregulate CD154 and rapidly migrate to lymph node
(4) (a) tgD-non specific and (b) tgD-specific (tgD+) B-cells access tgD-CD154 from DCs
(5) presentation of tgD to Th-cell by DC and B-cells via MHC-II
(6) activation of tgD+ B-cell by Th-cell and DC

vaccination, it may be important to carefully consider the biological activity of both the vaccine Ag and the CD154 molecule to ensure that the chimeric protein does not have competing receptor binding activities.

Acknowledgments

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