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ORIGINAL ARTICLE

RNA-loaded CD40-activated B cells stimulate antigen-specific T-cell responses in dogs with spontaneous lymphoma

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Cell-based vaccination strategies to induce functional tumor-specific T cells in cancer patients have focused on using autologous dendritic cells. An alternative approach is to use RNA-loaded CD40 activated B cells (CD40-B) that are highly efficient antigen-presenting cells capable of priming naive T cells, boosting memory T-cell responses and breaking tolerance to tumor antigens. The use of tumor RNA as the antigenic payload allows for gene transfer without viruses or vectors and permits major histocompatibility complex (MHC)-independent, multiple-antigen targeting. Here, we use CD40L transfected K562 cells to generate functional CD40-B cells from the peripheral blood of humans and dogs. Testing of RNA-loaded CD40-B cells in dogs allows not only for its development in veterinary medicine but also for determination of its safety and efficacy in a large animal model of spontaneous cancer prior to initiation of human clinical trials. We found that CD40-B cells from healthy humans, healthy dogs and tumor-bearing dogs express increased levels of immune molecules such as MHC and CCR7. Moreover, RNA-loaded CD40-B cells induce functional, antigen-specific T cells from healthy dogs and dogs with lymphoma. These findings pave the way for immunotherapy trials using tumor RNA-loaded CD40-B cells to stimulate antitumor immunity in a large animal model of spontaneous neoplasia.
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Introduction

The search for optimal vaccination methods to break tolerance to tumor antigens with the goal of eliciting clinically significant antitumor immune responses in cancer patients has been pursued over decades. Vaccination strategies using autologous dendritic cells (DC) to prime tumor-specific T cells and overcome aspects of immune incompetence in cancer patients have received the greatest attention.[1–3] The hypothesis is that prior to their injection in patients, *ex vivo* generated DC can be loaded with tumor-associated antigens which are then degraded by the proteasome into short peptides if necessary, transported into the endoplasmic reticulum, packaged in the groove of newly synthesized major histocompatibility complex (MHC) molecules and delivered as peptide-MHC (pMHC) complexes to the cell membrane. Engagement of the appropriate T-cell receptor (TCR) by these pMHC complexes conveys signals that, in the context of additional costimulatory interactions, activate the T cells. These activated tumor-specific T cells proliferate, produce cytokines and ultimately seek out and lyse tumor cells presenting the antigen.

Numerous clinical trials have evaluated the prospect of DC vaccines as a novel immunotherapy for patients with cancer.[4–8] Manufacturing issues however are not insignificant.[1] DC do not expand in culture, for example, and large volume leukapheresis is required to secure appropriate numbers of DC precursors to generate such vaccines.[9–11] As a consequence, application of DC-based vaccines in patients who cannot readily undergo leukapheresis (for example, women with breast cancer who have undergone mastectomy and axillary lymph node dissection or pediatric patients in whom large volume leukapheresis is not feasible) has been challenging.

An alternative approach to DC based vaccines is the use of CD40-activated B cells (CD40-B) as antigen presenting cells (APC).[12,13] Activation via CD40 enhances the antigen-presenting function of resting B cells, in large part by inducing high-level surface expression of MHC and co-stimulatory molecules.[12,13] When used with IL-4,

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CD40 activation causes marked B-cell proliferation *in vitro*, suggesting that unlike DC, a large number of CD40-B cells can be produced from a small number of precursors.[14,15] We have previously observed that 100–200 million CD40-B cells can be generated *ex vivo* from pediatric cancer patients in 3 weeks using only 4–8 ml of blood.[13] Furthermore, peptide-pulsed CD40-B cells are comparable to DC in their ability to stimulate antigen-specific cytotoxic T lymphocyte (CTL) responses[13] and when used *in vivo* have been shown to stimulate antitumor $CD8^+$ CTLs and delay tumor growth in a murine tumor model.[16] In previous work, we demonstrated that human CD40-B cells can be transfected with tumor antigen mRNA or whole tumor RNA to generate functional antitumor CTL *in vitro*.[13,17] The use of RNA as the antigenic payload in CD40-B cells allows gene transfer without the use of vectors or viruses and has the additional benefit of whole-antigen, MHC-independent targeting of tumor antigens. Taken together, these findings indicate that RNA-loaded CD40-B cells may represent a viable alternative to DC based vaccines in stimulating antitumor immunity.

Before evaluating RNA-loaded CD40-B cells in human patients, we have focused on *in vivo* studies to demonstrate the safety, immunogenicity and potential clinical efficacy of this approach. To do this, we have explored the prospect of treating privately owned animals that present with spontaneous tumors, such as dogs with lymphoma. The domestic dog shares a close phylogenetic relationship with humans and has been widely used to evaluate the safety and efficacy of gene transfer in the treatment of spontaneous genetic disorders.[18–22] Initial advancements in experimental bone marrow transplantation in research dogs paved the way for the translation of adoptive cellular therapies to human diseases.[23–25] These studies also demonstrated the utility of canine models for studying the immunobiology of hematopoietic cell transplantation. Privately owned, outbred domestic dogs that develop spontaneous cancers with similar biologic and behavioral characteristics to those in humans are a readily available and clinically relevant resource in which to study new therapeutic approaches to cancer, such as APC vaccination. Indeed, the evaluation of novel immunotherapies in canine cancer patients promises to provide more accurate preclinical data for human trials than rodent models, particularly as dogs are subject to the same environmental influences on tumor initiation and progression as their human counterparts and present with spontaneously occurring malignancies that have already evaded immune recognition.[26–28] Thus we hypothesize that canine cancer patients can provide a robust test of the *in vivo* clinical efficacy of therapies aimed at driving antitumor immune responses.

In this study, we demonstrate that large numbers of human and canine CD40-B cells expressing high levels of MHC class II can be generated *ex vivo* from small amounts of peripheral blood using a novel system of CD40-ligand (CD40L, also known as CD154) transfectants. Furthermore, CD40-B cells from healthy human and canine donors can be loaded with antigen-specific mRNA and used to stimulate functional, antigen-specific T-cell responses. Importantly, here we show that CD40-B cells can be generated from the peripheral blood of canine patients with spontaneously occurring lymphoma and these cells transfected with canine distemper virus hemagglutinin (CDV-HA) mRNA can stimulate canine distemper virus (CDV)-specific T-cell responses *in vitro*.

Results

Novel culture system for the generation of functional human CD40-B cells

Previously described methods to generate populations of human CD40-B cells rely on murine fibroblasts (NIH3T3) transfected with human CD40L.[12] This culture system is cumbersome, given that feeder cells are adherent and to date cannot be used under good manufacturing practice (GMP) conditions due to its xenogeneic nature. We subsequently modified this culture system with the use of clinical-grade recombinant human trimeric CD40L, but this reagent is no longer available from its manufacturer. To overcome these limitations, the human erythroleukemia cell line K562 was transfected with a hygromycin resistance expression plasmid containing human CD40L to produce an easily maintainable supply of CD40L expressing feeder cells (KtCD40L) in suspension. The parental K562 cells have the additional advantage of having previously been evaluated in human clinical trials of cell-based therapies. Co-culture of KtCD40L with healthy human donor peripheral blood mononuclear cells (PBMC) in addition to IL-4 and cyclosporine led to the generation of a highly enriched population of CD40-B cells that demonstrated robust expansion over five rounds of stimulation within a 30-day period (Figure 1a). Expansion results using suspension feeder cells (KtCD40L) were similar to those with adherent feeder cells (CD40L transfected murine NIH3T3 cells; Figure 1a). By day 12–14 in culture, the majority of cells generated with KtCD40L feeder cells expressed markers consistent with the B-cell lineage (CD19, CD79a and CD23) and expressed high surface levels of CD86 and MHC class II (Figure 1b and data not shown).

To determine whether mRNA-loaded human CD40-B cells generated with these culture conditions can induce functional T-cell responses *in vitro*, we first evaluated PBMC from HLA-$A2^+$ healthy donors co-cultured with autologous CD40-B cells electroporated with either GFP mRNA or mRNA from a *FluMP* mini-gene. This gene encodes an immunodominant influenza-derived epitope restricted to HLA-A2.[29] Following 7 days of stimulation of PBMC with *FluMP* mRNA-loaded CD40-B cells, but not GFP mRNA-loaded CD40-B cells, peptide/MHC tetramer analysis revealed marked expansion of *FluMP*-specific $CD8^+$ T cells (Figure 1c). Furthermore, *FluMP*-specific $CD8^+$ T cells stimulated with *FluMP* mRNA-loaded CD40-B cells efficiently and specifically lysed *FluMP* peptide-loaded T2 cells or SW40 tumor cells electroporated with *FluMP* mRNA (Figure 1d). Control T-cell cultures generated with GFP mRNA-loaded CD40-B cells exhibited minimal cytolytic activity against *FluMP*-loaded targets (Figure 1d). Furthermore, to demonstrate that the antigen-specific cytolytic T-cell responses elicited by *FluMP* mRNA-loaded CD40-B cells are MHC class I restricted, cytotoxicity assays were performed using *FluMP* peptide-loaded T2 target cells pre-incubated with the MHC class I specific monoclonal antibody W6/32 or control mouse immunoglobulin. The addition of W6/32 blocked *FluMP*-specific cytolytic

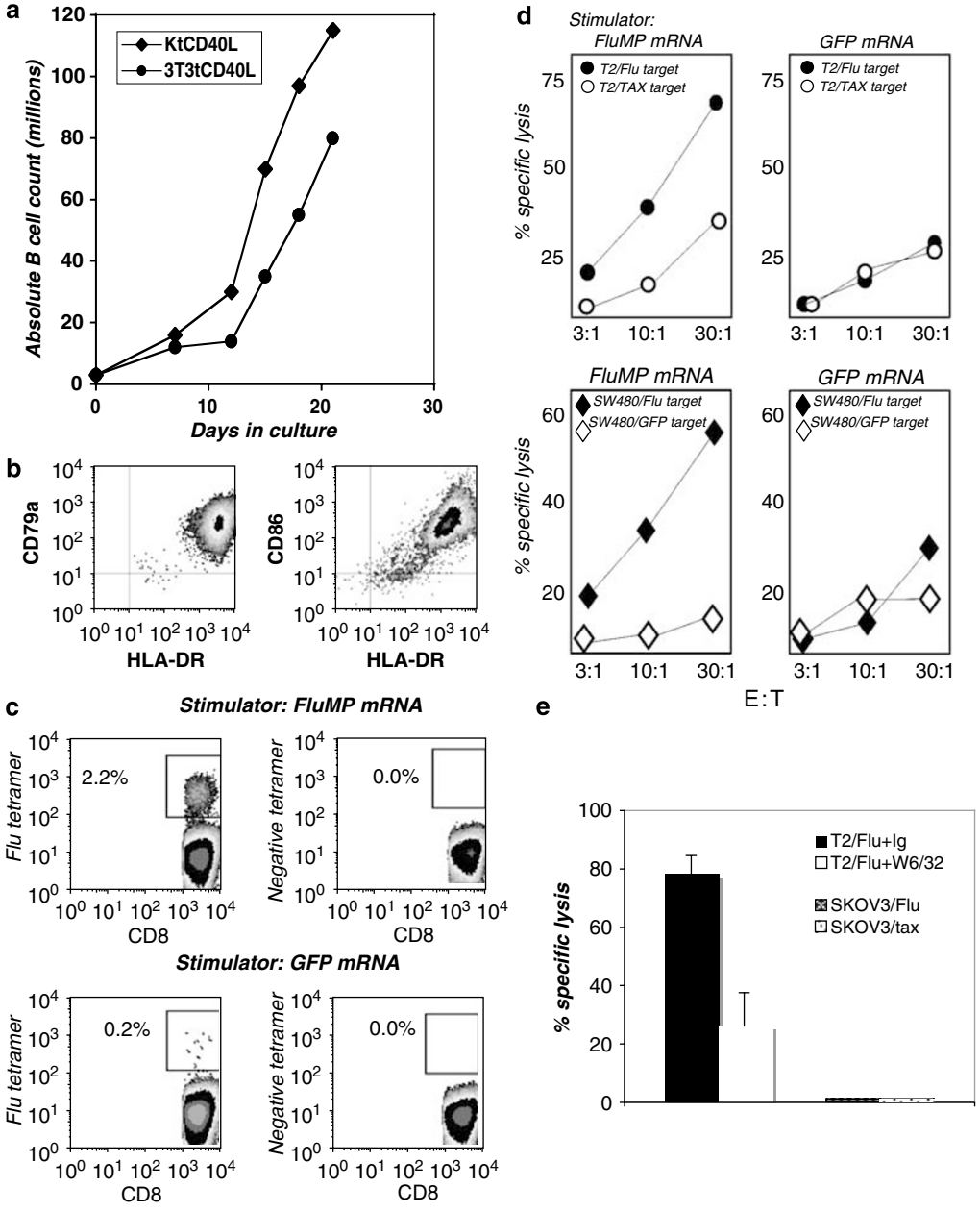

Figure 1 Generation of CD40-B cells using human CD40L K562 transfectants. (**a**) Growth curve comparison for two culture methods to generate human CD40-B cells. Peripheral blood mononuclear cells (PBMC) were stimulated with K562 cells transfected with human CD40L (KtCD40L; $n=4$; diamonds) or adherent 3T3 cells transfected with CD40L (3T3tCD40L; $n=3$; circles). One representative curve from each culture method is shown. (**b**) Day 14 CD40-B cells generated with KtCD40L cells were analyzed by flow cytometry for expression of CD79a, HLA-DR and CD86. Similar results were obtained from two healthy adult donors. (**c**) CD40-B cells from two healthy donors were electroporated with *FluMP* mRNA (upper panels) or GFP mRNA (lower panels) and co-cultured with autologous PBMC for 7 days. T-cell cultures were then harvested and stained with anti-CD8, anti-CD14, anti-CD4 and HLA-A2/Flu or HLA-A2/negative control tetramers. Cells were gated on $CD8^+$ $CD4^-$ $CD14^-$ mononuclear cells, and the percentage of $CD8^+$ tetramer + cells is indicated. (**d**) Autologous PBMC were stimulated once with CD40-B cells loaded with either *FluMP* mRNA (upper left and lower left panels) or with GFP mRNA (upper right and lower right) and were assayed for lysis of peptide-loaded T2 cells (upper panels: ● *FluMP* peptide; ○ tax peptide) or mRNA-loaded SW-480 carcinoma cells (lower panels: ♦ *FluMP* mRNA-loaded targets; ◇ GFP mRNA-loaded targets). SW-480 cells are $HLA\text{-}A2^+$ and MHC class II^-. Similar results were obtained with three donors. (**e**) Autologous PBL from an $HLA\text{-}A2^+$ donor were stimulated once with autologous CD40-B cells loaded with *FluMP* mRNA and were assayed for lysis of *FluMP* peptide-loaded T2 cells in the presence of 10 μg ml^{-1} W6/32 or 10 μg ml^{-1} of control mouse IgG and for lysis of the $HLA\text{-}A2^-$ SKOV3 cells loaded with either *FluMP* peptide or tax peptide. Experiments were performed in triplicate and s.d. of the triplicates is represented.

T-cell responses when compared to a control antibody (Figure 1e). As a further test of MHC restriction, we evaluated whether T cells elicited in an $HLA\text{-}A2^+$ donor by *FluMP* mRNA-loaded CD40-B cells could recognize $HLA\text{-}A2^-$ SKOV3 tumor cells. Minimal cytolytic activity by *FluMP*-specific $CD8^+$ T cells was observed against

SKOV3, loaded either with *FluMP* peptide or the HLA-A2-binding tax peptide from HTLV-1 (Figure 1e).

Canine CD40-B cells can be generated from healthy dogs and dogs with lymphoma

To determine whether CD40-B cells can be generated from canine donors using the same culture system, peripheral blood samples were obtained from privately owned healthy dogs and dogs with spontaneous lymphoma. PBMC were cultured with irradiated KtCD40L and recombinant canine IL-4 (rcIL-4) as described in Materials and methods. The substitution of human IL-4 with canine IL-4 was found to be required for optimal generation of canine CD40-B (cCD40-B) cells. From blood volumes of 4–5 ml (approximately 3–4 million PBMC, $2–5 \times 10^5$ naive B cells), 40–80 million cCD40-B cells were generated over 3–4 weeks of cell culture time from healthy dogs and those with lymphoma (Figure 2a). To determine the effect of KtCD40L on canine B cell proliferation, PBMC from healthy dogs and dogs with lymphoma were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with

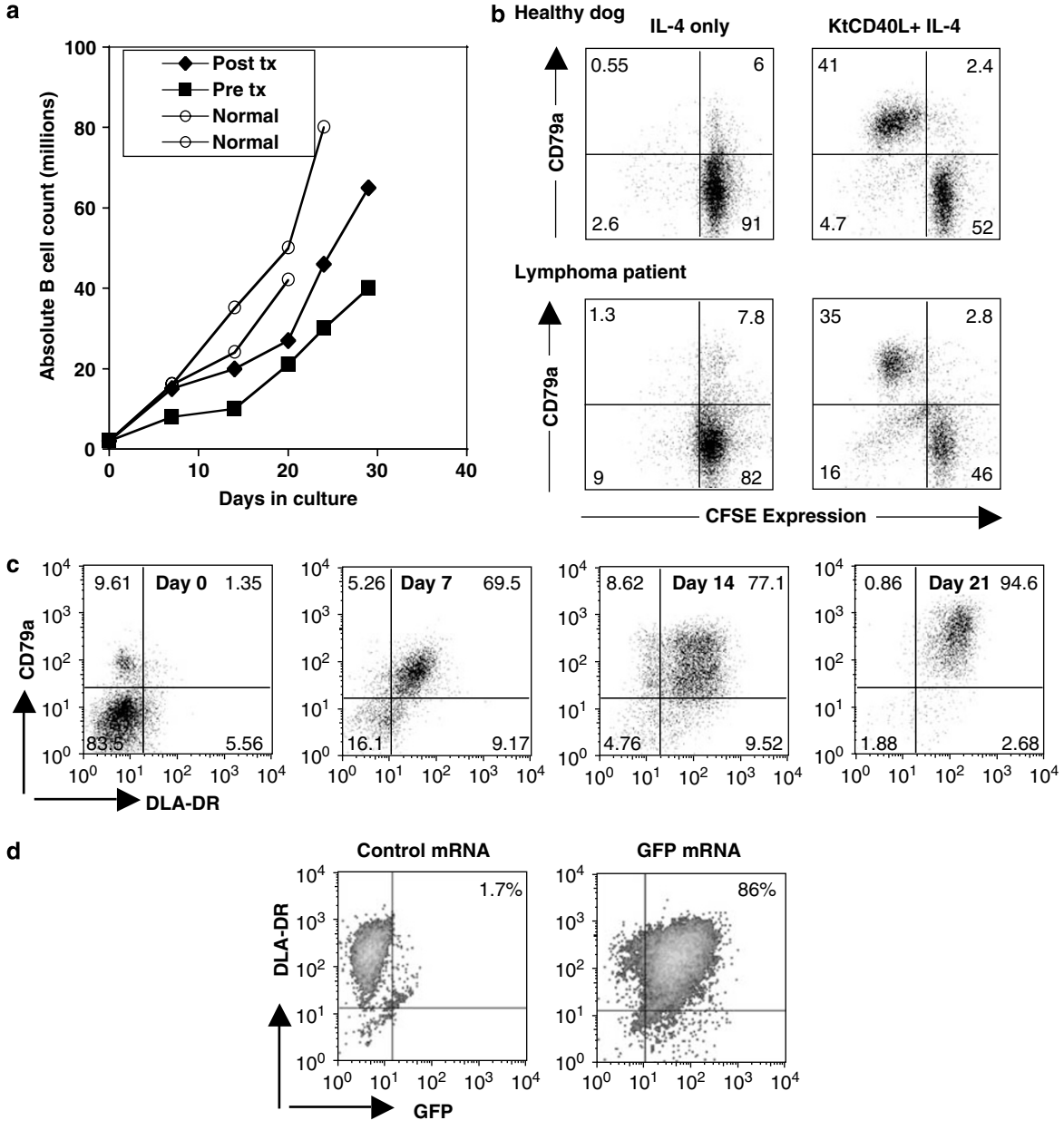

Figure 2 Generation of canine CD40-B (cCD40-B) cells. (**a**) Growth curve of CD40-B cells generated from canine peripheral blood mononuclear cells (PBMC; open circles, two healthy dogs; filled squares, one dog with lymphoma on chemotherapy; and filled diamonds, one dog with lymphoma post chemotherapy). (**b**) PBMC from a healthy dog (top panel) and a dog with lymphoma (bottom panel) were labeled with carboxyfluoroscein succinimidyl ester (CFSE) and stimulated with rcIL-4 with or without KtCD40L feeder cells. Cells were harvested on day 6 and analyzed for CFSE and CD79a expression. Similar results were obtained for two additional dogs with lymphoma. (**c**) cCD40-B cells generated from a dog with lymphoma were analyzed at baseline and weekly thereafter by flow cytometry for expression of DLA-DR and CD79a. Similar results were obtained for two additional dogs with lymphoma. (**d**) cCD40-B cells were electroporated with GFP mRNA or control (CDV-HA) mRNA and analyzed for GFP expression by flow cytometry 24 h later.

rcIL-4 in the presence or absence of KtCD40L. On day 6 of culture CD79a$^+$ cells from healthy dogs and dogs with lymphoma showed enhanced CFSE dilution in cultures containing KtCD40L + rcIL-4 when compared to cultures containing rcIL-4 alone (Figure 2b). Phenotypic analysis of cCD40-B cells using CD79a and MHC class II/dog leukocyte antigen-DR (DLA-DR) demonstrated a steady increase in the percentage of CD79a$^+$ DLA-DR$^+$ cells throughout the culture period (Figure 2c). At day 21 of culture flow cytometric analysis revealed that the majority of cells were CD79a$^+$ and expressed high levels of MHC class II/DLA-DR (Figure 2c). Very few contaminating CD3$^+$ T cells were present in the cultures. These results are similar to those obtained with human CD40-B cells generated using the same KtCD40L culture system. To determine whether cCD40-B cells can be efficiently transfected, cCD40-B cells were electroporated with GFP mRNA and analyzed for GFP and DLA-DR expression by flow cytometry. Transfection efficiencies of $>80\%$ were achieved in cCD40-B cells generated from both healthy canine donors and dogs with lymphoma (Figure 2d and data not shown). CD40-B cell viability after electroporation was similar in human and canine electroporated CD40-B cells ($>70\%$ at 24 h, data not shown).

cCD40-B cells loaded with CDV-HA mRNA elicit CDV-specific T-cell responses in healthy dogs

Routine vaccination of dogs against CDV generates CTL responses against the hemagglutinin (HA) antigen.[30–32] To determine whether mRNA-loaded canine CD40-B cells can induce functional T-cell responses *in vitro* against a viral antigen, cCD40-B cells were generated from three privately owned healthy dogs that had previously received the attenuated live CDV vaccine (Fort Dodge Animal Health, Kansas, IA, USA). cCD40-B cells harvested on day 14 of culture were electroporated with CDV-HA mRNA and co-cultured with autologous PBMC in the presence of rhIL-2 and rhIL-7. After 1 week of *in vitro* stimulation, T cells were analyzed by cIFN-γ ELISpot using CDV-HA mRNA-loaded autologous PHA blasts as target cells (Figure 3a). More IFN-γ secreting T cells were generated from PBMC stimulated with CDV-HA mRNA-loaded CD40-B cells when compared to those stimulated with control GFP mRNA-loaded CD40-B cells. Control T-cell cultures generated with GFP mRNA-loaded cCD40-B cells (negative control) did not secrete IFN-γ in response to any target cells.

cCD40-B cells loaded with CDV-HA mRNA elicit CDV-specific IFN-γ responses in dogs with spontaneous lymphoma

To determine whether functional T-cell responses can be generated *in vitro* in canine patients with spontaneously occurring lymphoma using autologous mRNA-loaded cCD40-B cells, cCD40-B cells were generated from a total of four dogs with spontaneous lymphoma, two dogs at the time of diagnosis and two dogs that had completed induction chemotherapy. The dogs studied at diagnosis were a cocker spaniel and a mixed breed with B-cell lymphoma. Dogs that had received chemotherapy were a Weimeraner and a mixed breed. The chemotherapy consisted of a CHOP-based protocol administered over 20 weeks on a cyclical weekly schedule and consisted

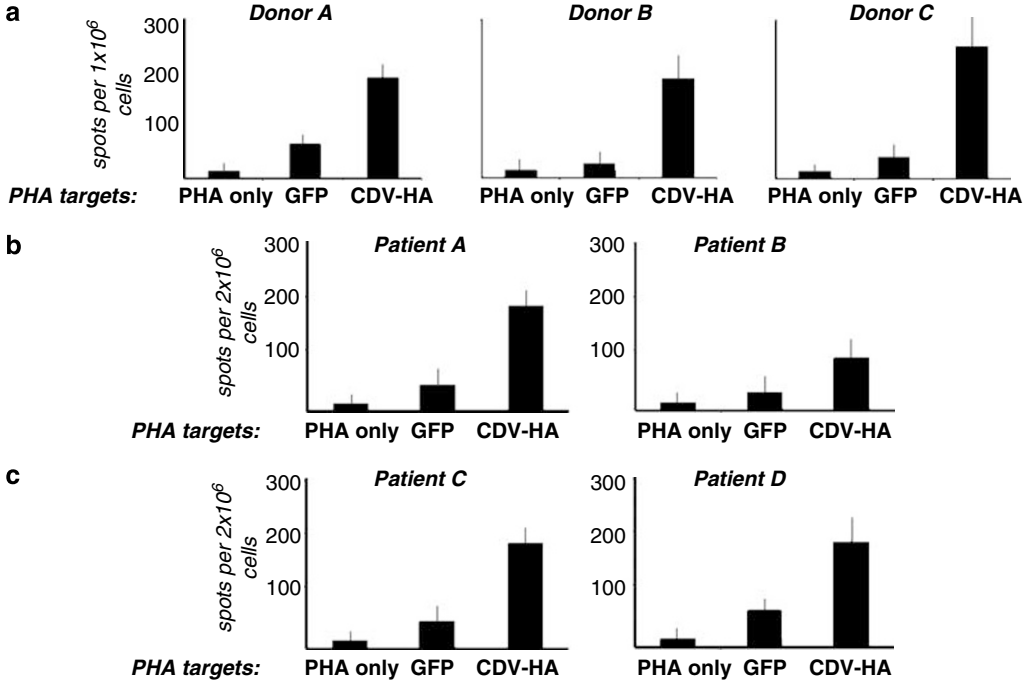

Figure 3 Canine CD40-B (cCD40-B) cells loaded with canine distemper virus hemagglutinin (CDV-HA) mRNA elicit CDV-specific IFN-γ responses in healthy dogs and dogs with spontaneous lymphoma. cCD40-B cells were generated from (**a**) three healthy dogs, (**b**) two dogs with lymphoma at the time of diagnosis and (**c**) two dogs with lymphoma during induction chemotherapy. All dogs had previously received the attenuated live CDV vaccine as a matter of routine veterinary care. cCD40-B cells harvested on day 14 of culture were electroporated with CDV-HA mRNA and co-cultured with autologous PBMC in the presence of rcIL-4, IL-7 and IL-2. On day 7, T cells were harvested and assayed for their ability to secrete IFN-γ in response to autologous PHA blasts electroporated with CDV-HA mRNA by cIFN-γ ELISpot assay. Experiments were performed in triplicate. Results for seven individual dogs (healthy donors A, B, C and patients A, B, C, D) are shown.

of L-asparaginase (400 IU kg^{-1} subcutaneously), vincristine (0.5 mg m^{-2} intravenously), cyclophosphamide (50 mg m^{-2} orally for 4 days) doxorubicin (30 mg m^{-2} intravenously) and a tapering dose of daily prednisone (starting at 2 mg kg^{-1} tapering over the first month). PBMC from these dogs were co-cultured with autologous cCD40-B cells loaded with either CDV-HA mRNA or GFP mRNA. T cells stimulated with CDV-HA mRNA loaded CD40-B cells were capable of secreting IFN-γ in response to target cells loaded with CDV-HA mRNA. The number of CDV-specific CTL secreting IFN-γ in response to GFP-loaded targets was less (Figure 3b). In two patients that had finished the chemotherapy protocol and where peripheral blood counts had returned to normal, the number of CDV-specific CTL secreting IFN-γ in response to CDV-HA-loaded targets was higher than in response to GFP-loaded targets (Figure 3c).

To further characterize the nature of the T-cell immune responses elicited by CDV-HA-loaded CD40-B cells, quantitative real-time PCR (qRT-PCR) analysis was performed on PBMC co-cultured with autologous cCD40-B cells loaded with CDV-HA mRNA. qRT-PCR demonstrated marked increases in IFN-γ and perforin expression, decreases in IL-10 and TGF-β expression and negligible changes in IL-4 expression following stimulation with CDV-loaded CD40-B cells when compared to unstimulated PBMC (Figure 4).

Canine CD40-B cells upregulate the lymphoid homing molecule CCR7

The ability of APCs to prime and activate antigen-specific T cells, once injected *in vivo*, depends upon their ability to home to secondary lymphoid organs where

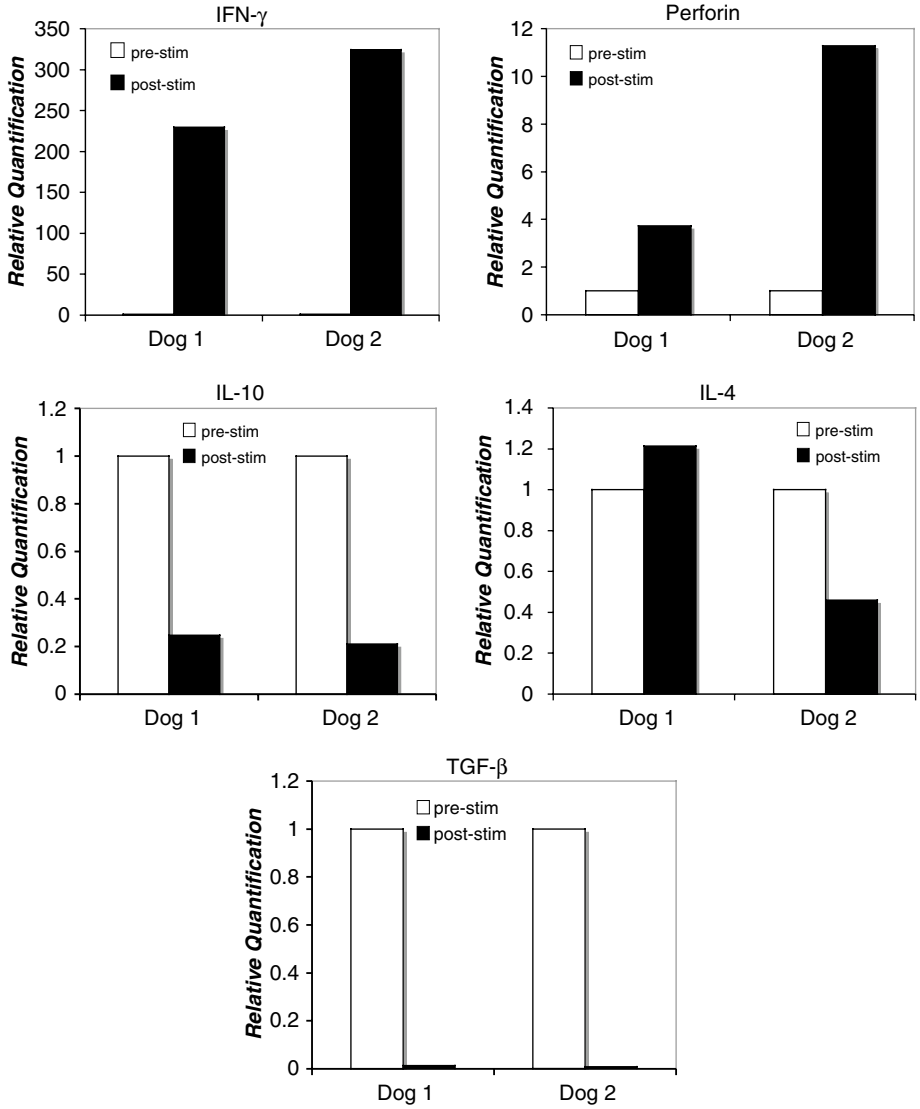

Figure 4 Cytokine expression of canine peripheral blood mononuclear cells (PBMC) stimulated *in vitro* with cCD40-B cells loaded with canine distemper virus hemagglutinin (CDV-HA) mRNA. Autologous PBMC were cultured with CDV-HA-loaded CD40-B cells in the presence of rcIL-4, IL-7 and IL-2. On day 7 of culture, cells were restimulated with CDV-HA-loaded CD40-B cells; 36 h later RNA was isolated and qRT-PCR analysis for IFN-γ, perforin, IL-4, IL-10 and TGF-β was performed. Values are expressed as relative quantification using either the 18S ribosomal subunit or β-actin as an endogenous control. RNA isolated from unstimulated PBMC was used for baseline assessments. Results are shown from two healthy dogs.

they interact with cognate naive and memory T cells. The chemokine receptors CCR7 and CXCR4 are upregulated on the surface of mature dendritic cells and mediate their chemotaxis to the lymph nodes.[33,34] Recent studies have demonstrated that these molecules are also upregulated on the surface of human CD40-B cells.[35] To determine whether canine B cells upregulate CCR7 following CD40 mediated activation, we first cloned and sequenced the canine CCR7 molecule and tested the ability of an anti-human CCR7 monoclonal antibody to cross-react with the canine molecule using flow cytometry. PCR amplification of cDNA obtained from canine CD40-B cells using canine CCR7-specific primers generated a product of 1137 bp. Sequence analysis revealed 100% homology to the predicted canine CCR7 sequence in GenBank (XM_548131). Translation of this product revealed an open reading frame of 1137 base pairs and a protein sequence that is 91% homologous to the human CCR7 sequence (Figure 5a). The product was cloned and transfected into the K562 cell line. Twenty-four hours later flow cytometric evaluation of these cells using a mouse anti-human CCR7 monoclonal antibody demonstrated the presence of a cross-reactive binding surface protein which was not detected in the control K562 cells transfected with the empty plasmid (Figure 5b). To determine whether canine B cells upregulate surface expression of canine CCR7 following activation with CD40L, canine CD79a$^+$ B cells were evaluated by flow cytometry before and after co-culture with KtCD40L and cIL-4. Following activation, canine CD40-B cells demonstrated a small but distinct shift in upregulation of surface CCR7 expression compared to isotype control mAb (Figure 5c), with maximal expression 24 h after CD40 ligand stimulation. These results are similar to those previously reported demonstrating the upregulation of CCR7 on human CD40-B cells.[35]

Discussion

The high-quality draft genome sequence of the dog has revealed its close phylogenetic relationship with human,[36] emphasizing the potential benefit of canine models in identifying disease genes and evaluating response to novel therapies. Unlike rodent xenograft models, spontaneous tumors that arise in outbred dogs mimic the biologic and behavioral characteristics of those that occur in humans.[28] Furthermore, dogs are subject to the same environmental influences on tumor initiation and progression as their human counterparts and

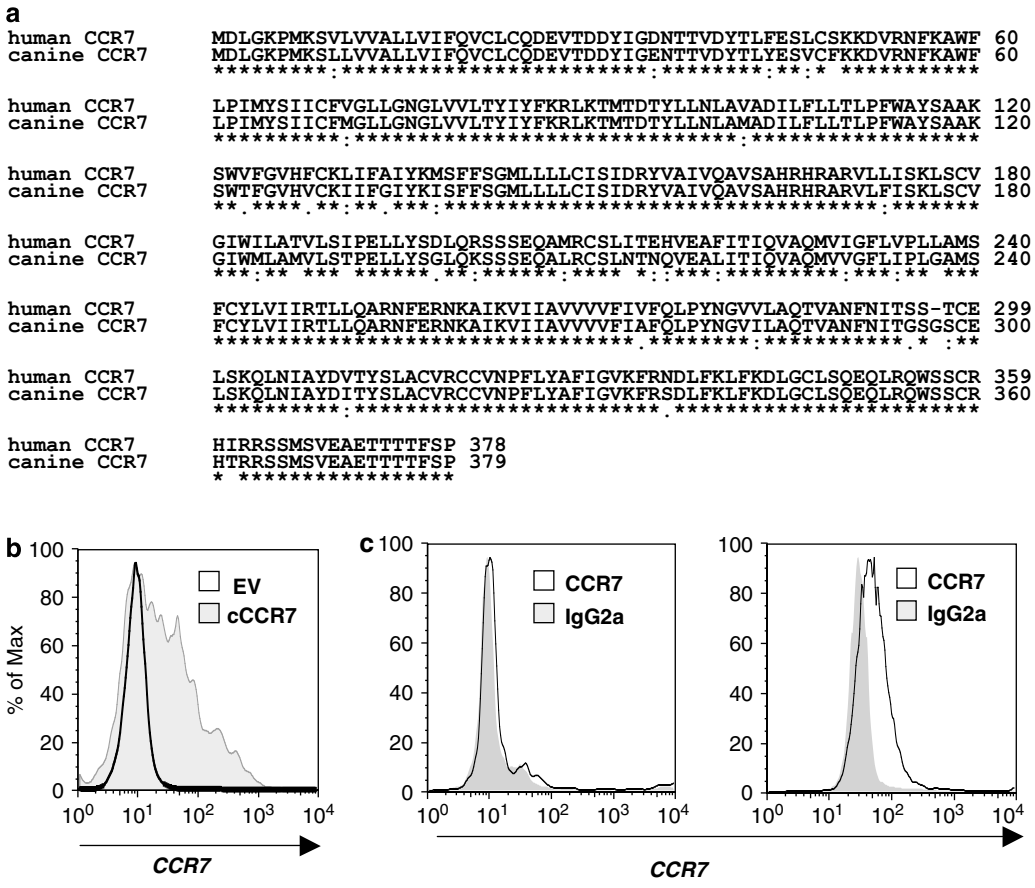

Figure 5 Molecular cloning and characterization of CCR7 expression on canine CD40-B cells. (**a**) Protein sequence alignments of cloned canine CCR7 and human CCR7. (**b**) K562 cells were transfected with either an empty vector (EV) or vector containing canine CCR7 and evaluated for surface expression of canine CCR7 using a mouse anti-human CCR7 monoclonal antibody. (**c**) Canine peripheral (unstimulated) B cells (left plot) and CD40-B cells (right plot) from 3 healthy dogs were labeled with CD79a and evaluated for surface expression of canine CCR7. Plots are gated on CD79a$^+$ cells. Open histogram, anti-CCR7 mAb; shaded histograms, mouse IgG2a isotype control mAb. Representative data from one dog are shown.

present with spontaneous malignancies that as in humans, have already evaded immune recognition or have actively suppressed antitumor immune responses. Thus, development of the CD40-B cell antigen presentation system as a cell-based vaccine for the treatment of the canine cancer patient was undertaken not only as a potential therapeutic in veterinary medicine but also to serve as a robust test of the safety and clinical efficacy of whole tumor RNA-loaded CD40-B cells in stimulating antitumor immune responses *in vivo* to an extent that is unparalleled in current rodent models.[28]

Previous work has shown that CD40-B cells generated using NIH-3T3 cells transfected with CD40L as feeder cells, and loaded with either antigen-specific RNA or whole tumor RNA, can induce functional T-cell responses *in vitro* against viral and tumor-associated antigens in pediatric and adult human oncology patients.[13] However, methods consistent with good manufacturing practice limit the use of xenogenic culture systems, and therefore, alternative systems to generate CD40-B cells are required. Attempts using research-grade human CD40L, CpG or toll receptor agonists combined with IL-4 have demonstrated initial proliferation of culture B cells, but have not been successful in generating the long-term culture stability and expansion that is seen with feeder cell expression of CD40L.[37] Here, we show that the human erythroleukemic cell line K562 can be stably transfected with human CD40L and used in a simple co-culture system to generate activated CD40-B cells that are comparable in their growth characteristics and phenotype to CD40-B cells generated using transfected NIH-3T3 cells.[12] The KtCD40L system has three advantages over NIH-3T3 transfectants; namely, the parental cells are of human origin, grow in suspension culture so are less cumbersome to harvest and require fewer restimulations to support long-term B-cell culture growth. Comparison of the amino-acid sequences of human CD40L (Swissprot no. P29965) and canine CD40L (Swissprot no. 097626) reveals that these sequences share a 79% amino-acid identity. The results reported here demonstrate that K562 cells transfected with human CD40L can stimulate canine B-cell activation and proliferation which is comparable to that achieved in human B-cell cultures and that the phenotype of cCD40-B cells are very similar to human CD40-B cells generated using the same system. Furthermore, we have shown that canine CCR7 is upregulated on the surface of canine CD40-B cells which is comparable with upreglation of CCR7 on human CD40-B cells.[35] This latter finding suggests that following the injection of these APCs *in vivo*, they should traffic to draining lymph nodes where they can interact with and efficiently activate antigen-specific T-cell responses. Taken together, these findings now allow for this technology to be translated into a spontaneous, large animal cancer model in order to test the safety and efficacy of RNA-loaded CD40-B cells to generate antitumor immunity *in vivo*.

The ability of human CD40-B cells to stimulate antigen-specific immune responses *in vitro* is generally accepted; however, the ability of CD40-B cells to stimulate antitumor immunity *in vivo* and to mediate clinical benefit in patients with spontaneously occurring cancer has not been evaluated. Based on this data, we have initiated a phase I clinical trial in which tumor RNA-loaded CD40-B cells are administered to privately owned, canine patients with spontaneously occurring lymphoma. The primary clinical endpoint is to evaluate safety and toxicity, and secondary endpoints include measuring the stimulation of antitumor immunity and impact on time to disease progression. Current therapy for canine lymphoma relies on combination chemotherapy, following which approximately 90% of dogs achieve complete remission. However, most ($>80\%$) canine patients relapse within 1 year and overall survival is less than 10% in most subtypes.[38] In our ongoing clinical trial, each dog enrolled receives autologous tumor RNA-loaded CD40-B cells and CDV-mRNA-loaded CD40-B cells (positive control) in the right and left flanks, respectively, once every 3 weeks for a total of three immunizations. To date, 21 canine patients with spontaneous lymphoma have been enrolled, and side effects have been minimal including mild constitutional signs and local injection site reactions.

The studies presented here provide proof of concept that RNA-loaded CD40-B cells can stimulate antigen-specific T-cell responses in canine patients with spontaneous lymphoma and thus pave the way to clinical trials evaluating the safety and potential clinical efficacy of RNA-loaded CD40-B cells in a clinically relevant, spontaneous, large animal cancer model. This trial will provide valuable clinical data necessary for the translation of this novel vaccination strategy to humans with cancer.

Materials and methods

Blood samples and cell lines

Peripheral blood was obtained from healthy adult human donors by leukapheresis after informed consent and with approval of the University of Pennsylvania Institutional Review Board. Blood samples were obtained by phlebotomy from healthy adult dogs or dogs diagnosed with lymphoma, after owner's informed consent and with approval of the University of Pennsylvania's Institutional Animal Care and Use Committee. All dogs had previously received routine CDV vaccination. PBMC from human or canine donors were isolated by Ficoll density gradient centrifugation. The human carcinoma cell lines SW-480 and SKOV3, the human erythroleukemia cell line K562 and the HLA-A2$^+$ transporter associated with antigen processing (TAP)-deficient human T2 cell line[39] were from the American Tissue Culture Collection (Manassas, VA, USA).

Generation of CD40-B cells using human CD40L K562 transfectants

K562 cells were transfected with the pcDNA3.1$^+$ expression plasmid containing human CD40L and a hygromycin resistance cassette. Transfected cells (KtCD40L) with >2 log mean fluorescence intensity of CD40L expression were sorted by flow cytometry and maintained under hygromycin selection, using culture conditions modified from previously published human culture conditions.[12,13] To generate CD40-B cells from human and canine PBMC, KtCD40L were lethally irradiated (96 Gy) and plated in six-well plates at 1×10^6 cells per well. Five million PBMC were resuspended in 4 ml of B-cell culture medium consisting of Iscove's modified Dulbecco medium (IMDM) supplemented with 10%

human AB serum, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), gentamicin (15 μg ml^{-1}) and insulin and transferrin (1:500 ITES supplement, Cambrex, East Rutherford, NJ, USA), and incubated with the irradiated KtCD40L in the presence of recombinant IL-4 (5 ng ml^{-1}; R&D Systems, Minneapolis, MN, USA; human or canine IL-4 was used depending on the source of PBMC) and cyclosporine (0.625 ug ml^{-1}; Novartis, Broomfield, CO, USA). On day 3, cultures were replenished with IL-4 and cyclosporine. After 5–7 days, CD40-B cell colonies were dispersed, resuspended in fresh B-cell media and restimulated with irradiated KtCD40L cells in the presence of IL-4 and cyclosporine. After the initial week in culture, cells were restimulated with fresh KtCD40L in fresh B-cell medium every 4–6 days.

Antibodies and flow cytometry

Human PBMC and CD40-B cells were analyzed for cell surface molecule immunophenotype as previously described.[13] Canine PBMC and CD40-B cells were analyzed by flow cytometry using the following antibodies: rat anti-canine CD3 (Serotec, Oxford, UK), rat anti-canine B cell (Serotec), rat anti-DLA-DR (Serotec), mouse anti-human CD79a (BD PharMingen, San Diego, CA, USA) and mouse anti-human CCR7 (R&D Systems). For intracellular staining, cells were fixed and permeabilized (Fix and Perm solution, Caltag Laboratories, San Francisco, CA, USA) prior to staining. In experiments to analyze KtCD40L-induced B-cell proliferation, canine PBMC were washed twice with PBS, resuspended to a concentration of 10×10^6 ml^{-1} and labeled with CFSE (Molecular Probes, Eugene, OR, USA) 1.25 μM for 5 min at room temperature. Labeling was quenched with 5% FCS and cells were washed three times in B-cell media prior to stimulation with KtCD40L, as described above.

mRNA preparation

mRNA encoding full-length *CDV-HA* or *eGFP* genes was prepared from template plasmids containing the full-length cDNA (pCIneo-CDV-HA or pVA67D-eGFP) by *in vitro* transcription using the T7 RNA polymerase promoter and the mMessage mMachine kit (Ambion, Austin, TX, USA). Transcribed RNA was polyadenylated at the 3′ terminus using the *Escherichia coli* Poly(A) Polymerase I (E-PAP), treated with DNase to remove plasmid sequences and purified by acidic phenol/chloroform extraction and RNeasy column separation (Qiagen, Valencia, CA, USA). Measurement of OD_{260} and formaldehyde denaturing gel electrophoresis were used to determine quantity and quality of mRNA.

Establishment of T-cell cultures using RNA-electroporated CD40-B cells

For RNA electroporation of CD40-B cells, CD40-B cells were harvested after 2 weeks of culture, washed twice in PBS, resuspended at 2–2.5 $\times 10^6$ per 100 μl of Nucleofector B solution (Amaxa, Cologne, Germany) and electroporated with 2 μg mRNA/sample (pulse program U08). Expression of electroporated mRNA in CD40-B cells was determined by GFP expression 24 h after electroporation. Electroporated human or canine CD40-B cells were then washed in T-cell medium (RPMI with 10% human AB serum, 2 mM glutamine, 20 mM HEPES and 15 μg ml^{-1} gentamycin) and plated at 5×10^5 cells per well with 3×10^6 PBMC/well in 24-well plates. Cultures were supplemented with 500 U ml^{-1} rhIL-4 or rcIL-4 and 10 ng ml^{-1} rhIL-7 (Sigma, St Louis, MO, USA) on day 0 and 20 U ml^{-1} rhIL-2 (Chiron, Emeryville, CA, USA) on days 1 and 4.

Chromium^{51} release assay

Chromium release assays were performed as previously described.[40] Target cells included T2 cells loaded with 10 μg ml^{-1} peptide (New England Peptide, Fitchburg, MA, USA) and 2.5 μg ml^{-1} β2-microglobulin (Sigma) and SW480 cells loaded with 10 μg ml^{-1} peptide for evaluation of human CTL activity. The HLA-A2-binding HTLV-1 L11 tax peptide served as a negative control peptide. Standard deviation was less than 5%. For MHC class I blocking studies, the MHC-specific monoclonal antibody W6/32 (Serotec) and control mouse IgG antibody (Jackson Immuno Research, West Grove, PA, USA) were used at a final concentration of 10 μg ml^{-1}. Antibodies were incubated with target cells for 30 min at 37 °C before the addition of T cells.

Tetramer analysis

Peptide/MHC tetramer for the HLA-A2-restricted dominant epitope of *FluMP* (GILGFVFTL) and negative control HLA-A2 tetramer were purchased from Immunomics (San Diego, CA, USA). Peptide/MHC tetramer analysis was performed as previously described.[13]

ELISPOT analysis

For IFN-γ analysis, T-cell cultures at 2.5×10^4 cells per well were added to ImmunoSpot plates (Cellular Technology, Cleveland, OH, USA) precoated with 10 μg ml^{-1} anti-IFN-γ mAb (Mabtech, Nacka, Sweden) in the presence of stimulator cells overnight at 37 °C. Stimulator cells included T2 cells at 5×10^5 cells per well with 5 μg ml^{-1} peptide and 1 μg ml^{-1} β2-microglobulin. After 24 h at 37 °C, wells were developed as previously described[41] and were counted using a Prior ProScan analyzer and Image Pro Plus software (Hitech Instruments, Edgemont, PA, USA). For IFN-γ analysis of CD40-B cell-stimulated canine T cells, autologous PBMC were stimulated with phytohemagglutinin (PHA (5 μg ml^{-1}); Sigma) and rhIL-2 for 5 days, electroporated with CDV mRNA or GFP mRNA and used as targets in co-cultures with RNA-loaded CD40-B cell-stimulated T cells (2.5×10^4 cells per well). ELISPOT analysis was performed using the canine IFN-γ ELISPOT kit to detect antigen-specific responder cells (R&D Systems).

Quantitative real-time PCR evaluation

Total RNA was extracted from T cells stimulated with CDV-HA-loaded CD40-B cells 36 h after restimulation, using the Qiagen RNeasy Isolation System (Qiagen). Reverse transcription was performed using random hexamers and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad. CA, USA) according to the manufacturer's instructions. Taqman PCR primers and probes for canine IFN-γ, IL-4, IL-10, TGF-β and the eukaryotic 18S ribosomal protein were obtained from Applied Biosystems (Foster City, CA, USA). Transcript sequences for canine perforin (XM_546148) were obtained from the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and

were analyzed for secondary DNA structure using M-fold[42] (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi. Primers for canine perforin were designed using Primer 3 software (http://frodo.wi.mit.edu) with the maximum self-complementarity score set at 5 and the maximum 3 self-complementarity score set to 0 to minimize primer-dimer formation. Primers were synthesized by Invitrogen Corp. The following primer sequences were used in qRT-PCR analysis: canine perforin forward, 5′-CCAAGACTAT GGCTGGGATG-3′; canine perforin reverse, 5′-GCAC TTGGCAATGTAGGAGAA-3′. Annealing temperatures of all primers were 60 °C. qRT-PCR primers used for canine β-actin have been previously described.[43] Real-time PCR assays for canine perforin and β-actin were performed using SYBR Green I based detection (Applied Biosystems). SYBR Green and Taqman assays were run in triplicate at standard conditions using a sequence detector (ABI 7500; Applied Biosystems) and data were analyzed on Taqman Assays on Demand (Applied Biosystems). Taqman assays utilized the 18S ribosomal subunit as an endogenous control and SYBR Green assays utilized β-actin as an endogenous control. Dissociation curves were performed after each experiment that used SYBR green technology to confirm the specificity of product amplification and ensure that false positive results were not obtained.

Cloning and characterization of canine CCR7

Total RNA was extracted from canine CD40-B cells using RNA Stat 60 (IsoTex Diagnostics, Inc. Friendswood, TX, USA) according to the manufacturers' instructions. Reverse transcription was performed using random hexamers and the first strand cDNA synthesis system (Amersham Pharmacia Biotech, Little Chalfont, England). Forward and reverse primers for canine CCR7 were designed based on the predicted sequence for CCR7 (PubMed XM_548131) with BAMHI and XHOI restriction sites incorporated into the forward and reverse primers respectively (cCCR7 forward primer: 5′-GGATC CATGGACCTGGGGAAACCAATGAAAAG-3′; cCCR7 reverse primer: 5′-CTCGAGCTACGGGGAGAAGGTGG TGGTGGTC-3′). Cycling conditions were as follows: denaturation, 94 °C for 3 min; annealing, 64 °C for 30 s; extension, 72 °C for 3 min. PCR products were cloned into the pcDNA3.1$^+$ expression vector and sequenced. K562 cells were transfected with the empty pcDNA3.1$^+$ plasmid or pcDNA3.1$^+$ containing canine CCR7 using the Amaxa transfection system (Amaxa). Twenty-four hours later, cells were analyzed for the surface expression of canine CCR7 using a monoclonal mouse anti-human CCR7 (clone 150503; R&D Systems).

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