

# Agonistic CD200R1 DNA Aptamers Are Potent Immunosuppressants That Prolong Allogeneic Skin Graft Survival

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CD200R1 expressed on the surface of myeloid and lymphoid cells delivers immune inhibitory signals to modulate inflammation when engaged with its ligand CD200. Signalling through CD200/CD200R1 has been implicated in a number of immune-related diseases including allergy, infection, cancer and transplantation, as well as several autoimmune disorders including arthritis, systemic lupus erythematosus, and multiple sclerosis. We report the development and characterization of DNA aptamers, which bind to murine CD200R1 and act as potent signalling molecules in the absence of exogenous CD200. These agonistic aptamers suppress cytotoxic T-lymphocyte induction in 5-day allogeneic mixed leukocyte culture and induce rapid phosphorylation of the CD200R1 cytoplasmic tail thereby initiating immune inhibitory signalling. PEGylated conjugates of these aptamers show significant *in vivo* immunosuppression and enhance survival of allogeneic skin grafts as effectively as soluble CD200Fc. As DNA aptamers exhibit inherent advantages over conventional protein-based therapeutics including low immunogenicity, ease of synthesis, low cost, and long shelf life, such CD200R1 agonistic aptamers may emerge as useful and safe nonsteroidal anti-inflammatory therapeutic agents.

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## Introduction

Controlled modulation of the immune system using agonistic or antagonistic ligands targeting costimulatory or coinhibitory cell surface receptors offers great potential for the treatment of diseases including allergy, infection, autoimmune syndromes, transplantation, heart disease, and cancer. One such receptor, CD200R1, a type I glycoprotein expressed on cells of myeloid and lymphoid lineage, delivers immune inhibitory signals upon ligation to the widely distributed cell surface glycoprotein CD200.<sup>1–3</sup> Structurally, CD200R1 contains two Ig-like domains, a transmembrane region, and a cytoplasmic tail containing a NXPY motif which is phosphorylated upon CD200 ligation inducing the recruitment of the adaptor protein Dok2 and subsequent signal transduction events.<sup>4–6</sup>

The physiological importance of CD200:CD200R1 inhibitory signalling has been established in a number of diseases including arthritis,<sup>7–10</sup> transplantation,<sup>11–15</sup> as well as central nervous system autoimmune diseases such as Parkinson's disease and multiple sclerosis.<sup>16–21</sup> A recombinant CD200Fc fusion protein has been shown to behave as a potent *in vivo* immunosuppressant, prolonging allo- and xenograft survival<sup>11,22</sup> as well as suppressing collagen-induced arthritis in mice.<sup>7</sup> Also, the inhibition of CD200:CD200R1 signalling on microglial cells using a blocking antibody to CD200R1 exacerbated neurodegeneration and disease state in a murine model of experimental autoimmune encephalomyelitis.<sup>23</sup> These findings were further supported in a separate experimental autoimmune encephalomyelitis study where treatment with CD200Fc suppressed microglial accumulation,

and decreased the production of proinflammatory cytokines IL-6, TNF- $\alpha$ , and nitric oxide by myeloid cells in the spleen and central nervous system.<sup>24</sup> CD200R1 signalling has been implicated in tissue specific autoimmunity as well, as both systemic and local treatment with an anti-CD200R1 agonistic antibody suppressed experimental autoimmune uveitis, a model of CD4<sup>+</sup> T-cell organ-specific autoimmunity of the eye.<sup>25</sup> Thus, the development of safe and effective immunomodulatory agents which stimulate CD200R1 signalling are of clinical interest.

Aptamers are short single-stranded nucleic acids (RNA or ssDNA) that can be readily developed to bind a molecular target of interest with affinity and specificity features which compare well with monoclonal antibodies. As in the case of antibodies, aptamers can be derived to either block protein–protein interactions or act as agonists to cell surface receptors, suggesting the use of such functional aptamers as therapeutic agents.<sup>26–28</sup> In contrast to antibodies and other protein-based agents, aptamers have a number of advantages including a long shelf life, low immunogenicity, and cost-effective scalable chemical synthesis.<sup>26–28</sup> However, aptamers as therapeutic entities do display poor pharmacokinetic profiles as unprotected RNA or DNA aptamers are rapidly removed from circulation due to renal filtration and nuclease degradation.<sup>27</sup> Their pharmacokinetic properties can be improved upon site-specific conjugation of polyethylene glycol (PEG) polymers to aptamer termini to reduce renal filtration as well as the incorporation of nuclease resistant 2'-F or 2'-O-Me nucleotides in the case of RNA aptamers to impart nuclease resistance.<sup>27,29,30</sup> Functional aptamers which

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target costimulatory or coinhibitory receptors represent a new class of targeted immunotherapeutic agents with unique and advantageous properties. Thus far, aptamers with either agonistic or antagonistic function have been developed to a number of immune receptors including CTLA-4,<sup>31</sup> 4-1BB,<sup>32</sup> OX-40,<sup>33,34</sup> IL-6R,<sup>35</sup> IL-10R,<sup>36</sup> and CD28<sup>37</sup> with only a few of them being validated for activity *in vivo*.<sup>31–33,36,37</sup>

Here we report the development and characterization of two DNA aptamers, which bind to murine CD200R1 and stimulate immune inhibitory signalling. These agonistic aptamers, termed M49 and M52, exhibit *in vitro* immunosuppressive properties as measured by their ability to suppress cytotoxic T-lymphocyte (CTL) induction in allogeneic-mixed lymphocyte cultures (allo-MLC). Importantly, PEGylated conjugates of these aptamers retain their immunosuppressive function both *in-vitro* and *in-vivo*. Furthermore, we demonstrate the therapeutic potential of agonistic CD200R1 aptamers as the intravenous administration of PEG-M49 and PEG-M52 prolongs the survival of murine skin allografts to a similar extent as CD200Fc.

## Results

### Generation of CD200R1-specific DNA aptamers displaying agonistic signalling properties

Over 20 DNA aptamer sequences specifically recognizing a murine CD200R1 recombinant protein were identified after 15 rounds of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) screens. These 75-base long sequences along with a scrambled control aptamer (cApt) (Figure 1a) were synthesized and systematically screened for CD200R1 agonistic activity by evaluating their ability to suppress the induction of CTL in a 5 day allo-MLC assay. Aptamer-induced suppression of CTL induction was monitored using a chromium release assay of loaded P815 mastocytoma cells serving as target cells for CTL lysis. Four aptamers termed M21, M48, M49, and M52 (Figure 1a) displayed CD200R1 agonistic properties (Figure 1b). Specifically, aptamers M49 and M52 suppressed CTL induction at levels comparable to that of a soluble CD200Fc ligand with less than 5% CTL specific lysis of P815 cells occurring at aptamer concentrations  $\geq 325$  nmol/l. M49 and M52 were chosen for further evaluation. The binding affinity of each aptamer to the murine CD200R1 extracellular domain was derived using a nitrocellulose filter retention assay. M49 bound to CD200R1 with a dissociation constant ( $K_D$ ) of  $390 \pm 86$  nmol/l while the  $K_D$  for M52 interacting with its target was estimated to be  $>1$   $\mu$ mol/l; a value comparable with the reported binding affinity of mCD200 to mCD200R1 of 4  $\mu$ mol/l.<sup>38</sup>

The 75-base long M49 and M52 aptamer sequences were truncated based on their predicted secondary structure derived from mfold<sup>39</sup> software (Figure 2a). M49 retained agonistic activity when truncated to a minimal size of 55 nucleotides while the optimal activity for M52 was retained down to a length of 44 nucleotides (Figure 2b).

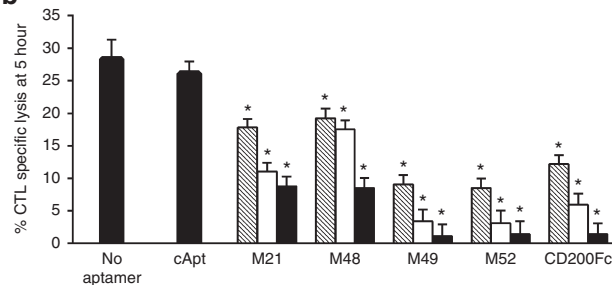
### PEGylation of M49 and M52

Conjugation of aptamers to high molecular mass polymers such as polyethylene glycol (PEG) has been shown to dramatically improve their circulating half-lives from 5–10 minutes

a

Name	Sequence
M21	CCGCTACCCATCCAGCCAAATCC
M48	GCAGATGGATGATCAGGCTATTTCC
M49	GACGTGACATGCTTGACCAACTCGC
M52	TTTATTACCATTATGCCTATGTAA
cApt	TCCCGCATCCTCCGCGTCCCGACC

b



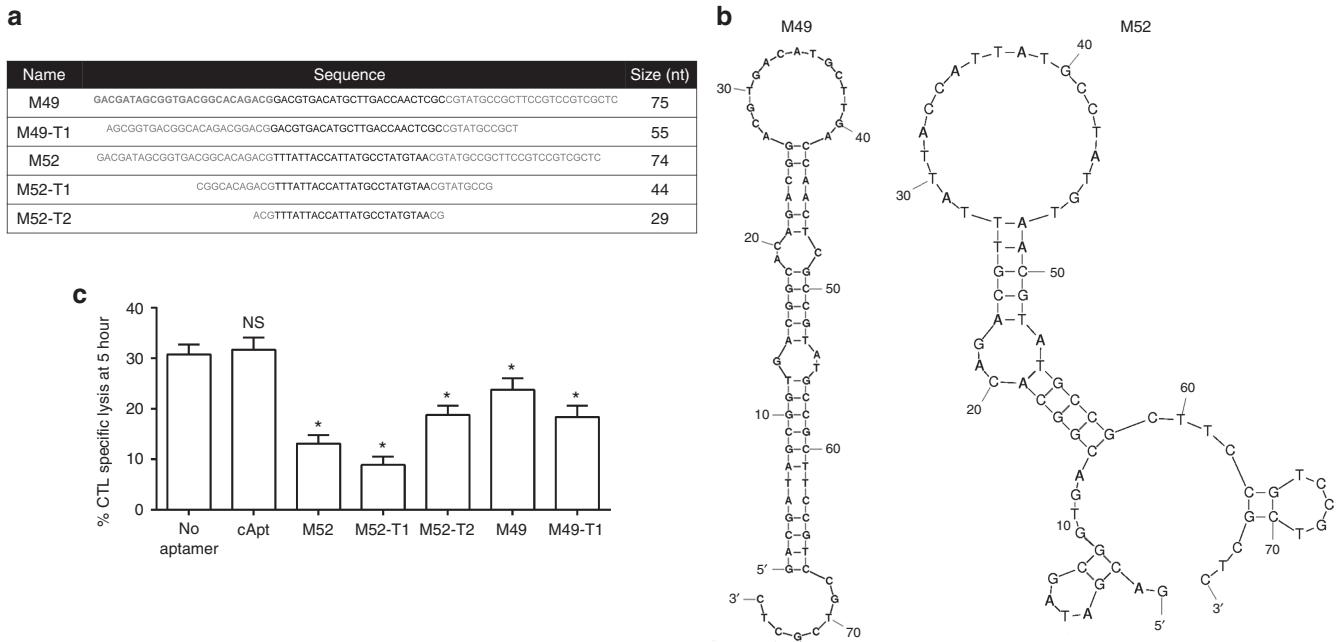
**Figure 1** DNA aptamers selected to bind to the extracellular domain of murine CD200R1 suppress cytotoxic T lymphocytes (CTL) induction in primary allo-MLC. (a) Table of selected aptamer sequences identified after 15 iterative SELEX rounds. Full-length aptamers consist of an internal variable region of 25nt, flanked by two constant regions of 25nt each. A control aptamer (cApt) composed of a randomly selected variable sequence and the same constant regions present in the library was used throughout this study. (b) DNA aptamers were added to 5-day allo-MLC with  $2.5 \times 10^5$  C57BL/6 responder cells incubated with an equal number of irradiated BALB/c stimulator cells. Induction of CTLs was monitored by the release of <sup>51</sup>Cr from P815 mastocytoma target cells at a 25:1 effector-to-target ratio. Soluble CD200Fc was used as a positive control. Each bar represents the mean percent of CTL-specific lysis  $\pm$  SEM from three replicate wells. Aptamers were added at 975 nmol/l (closed bars), 325 nmol/l (open bars), or 110 nmol/l (hatched bars). \* $P < 0.01$  when compared to cApt or no treatment.

to as long as 1–2 days.<sup>27,29,30</sup> Thus, a 20 kDa monofunctional PEG moiety carrying an N-hydroxysuccinimide ester was thus reacted with a 5' hexylamine arm introduced during the synthesis of DNA aptamers M49 and M52 (Supplementary Figure S1a). The resulting PEGylated M49, M52, and cApt conjugates were recovered by ultrafiltration and further purified by size exclusion chromatography. The purity of the final PEGylated products were confirmed by size exclusion fast protein liquid chromatography (Supplementary Figure S1b) and polyacrylamide gel electrophoresis (data not shown).

### PEGylated M49 and M52 retain agonistic CD200R1 function

To ensure that the PEGylation of DNA aptamers M49 and M52 did not disrupt their folding and agonistic activity, these modified aptamers as well as PEG-cApt were compared to unconjugated aptamers for their ability to suppress CTL induction in an allo-MLC assay. Interestingly, both PEG-M49 and PEG-M52 suppressed CTL induction to a greater extent than M49 and M52 (Figure 3a) confirming that PEGylation did not disrupt their immunosuppressive function.

The immediate signalling event following CD200:CD200R1 ligation is the phosphorylation of the tyrosine residue in the NPXY motif on the C-terminal cytoplasmic tail of CD200R1.<sup>6,7</sup> The phosphorylated NPXY motif interacts with adaptor proteins thereby transducing immune inhibitory signalling.<sup>4–6</sup> To



**Figure 2 Truncation of CD200R1 aptamers M49 and M52.** (a) Table of aptamer sequences truncated based on secondary structure predictions. Shown is aptamer name, sequence, and length in nucleotides (nt). The constant primer regions on each sequence are highlighted in red. (b) Secondary structure predictions derived from mfold<sup>39</sup> were used to guide truncation of M49 and M52. (c) Truncated aptamers were added to 5-day allo-MLC and suppression of CTL induction assayed as described. Truncated sequences M52-T1 and M49-T1 suppress CTL induction with comparable efficacy to that of the full-length aptamers. Each bar represents the mean percent CTL specific lysis  $\pm$  SEM from three replicate wells and is representative of two independent experiments (\* $P < 0.01$ , NS, not significant).

confirm that the suppression of CTL induction observed in our allo-MLC assays is indeed a consequence of aptamer-induced CD200R1 signalling, we asked whether PEG-M49 and PEG-M52 could induce the phosphorylation of this motif. HEK-293 cells were stably transfected to express murine CD200R1 (Figure 3b) and treated with aptamers PEG-M49 and PEG-M52. The phosphorylation of CD200R1 was detected using a phosphospecific antibody. Both PEG-M49 and PEG-M52 induced the rapid phosphorylation of the C-terminal tail of CD200R1 (Figure 3c). There was no detectable signal from medium alone or PEG-cApt confirming that the identified aptamers signal through CD200R1 in a similar manner to CD200.

### PEGylated CD200R1 aptamers suppress immune responses *in vivo*

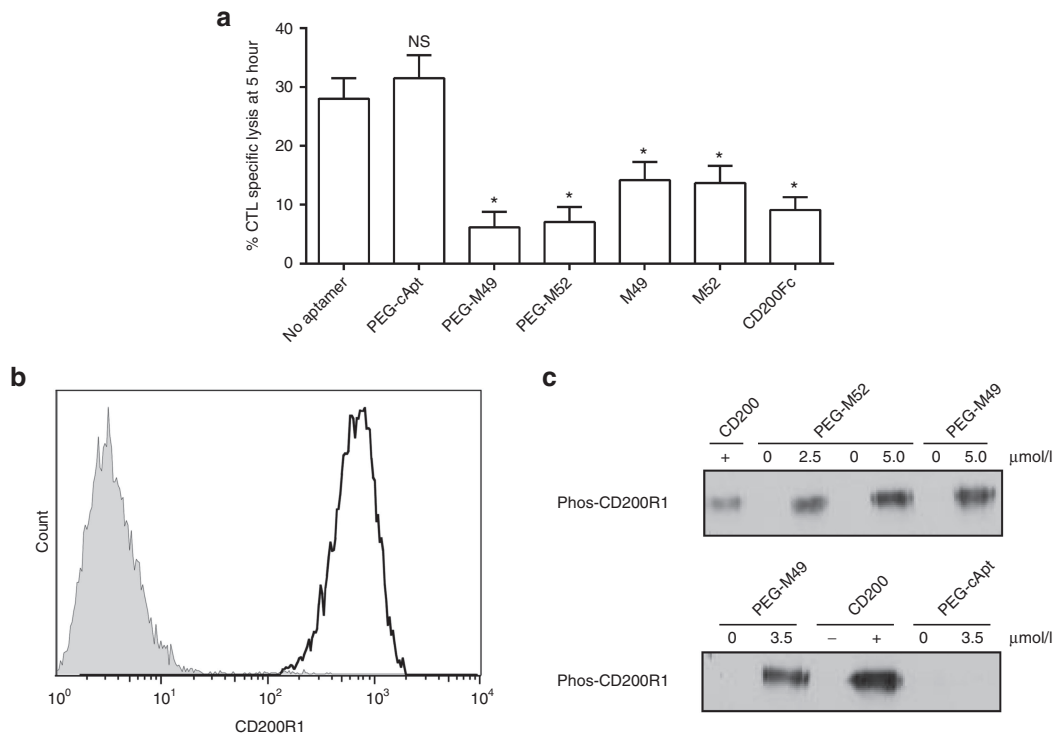
PEG-M49, PEG-M52, PEG-cApt, or CD200Fc were injected intravenously into C57BL/6 mice harboring skin allografts with a view to evaluate the potential *in vivo* therapeutic use of the CD200R1 agonistic DNA aptamers. PEGylated aptamers were administered at 72-hour intervals over 12 days and their immune inhibitory effects evaluated by performing *ex vivo* allo-MLC assays at day 14 (Figure 4a). CTL induction was significantly suppressed by treating animals with PEG-M49, PEG-M52, or CD200Fc but not with PEG-cApt ( $P < 0.05$ ) (Figure 4b). Exposure of circulating lymphocytes to PEG-M49 and PEG-M52 both *in vivo* and after their recovery (*in vitro*) did not further augment the suppression of CTL induction as compared to the *in vivo* treatment alone (Figure 4b).

A common concern regarding the *in vivo* use of DNA aptamers is the potential induction of TLR9-mediated innate immune activation similar to oligodeoxynucleotides containing CpG motifs. To address whether PEG-M49 or PEG-M52 induce innate TLR9 responses, naive C57BL/6 mice were administered aptamers or a CpG oligonucleotide (ODN) and TNF- $\alpha$  and IL-6 sera levels quantified after six hours by ELISA. Only the control CpG ODN exhibited detectable cytokine levels (Supplementary Figure S2) suggesting that these aptamers do not activate a TLR9-mediated innate immune response in mice.

Together, these findings suggest that the *in vivo* administration of PEGylated CD200R1 agonistic aptamers is sufficient to downregulate immune responses and that such aptamers may serve as anti-inflammatory agents for diseases in which CD200:CD200R1 signalling is implicated.

### PEGylated CD200R1 aptamers prolong allogeneic skin graft survival

The importance of CD200:CD200R1-based immune regulation in transplantation is well documented. Soluble CD200Fc administration has been shown to significantly prolong the survival of both allo- and xenografts.<sup>11,22</sup> Moreover, transgenic mice overexpressing CD200 display prolonged allograft survival rates relative to control animals. In contrast, CD200R1 knockout mice reject allografts more vigorously.<sup>14</sup> Lastly, a soluble form of CD200 (sCD200) with immunosuppressive function has been observed in the sera of mice after transplantation with serum levels correlating positively with graft survival.<sup>15</sup> Therefore, we hypothesized that the administration



**Figure 3** PEG-conjugated M49 and M52 function act as CD200R1 agonists. (a) PEGylated and non-PEGylated aptamers (325 nmol/l, based on nucleic acid content) were added to 5-day allo-MLC and suppression of cytotoxic T lymphocytes (CTL) induction assayed. PEG-M49 and PEG-M52 significantly suppress CTL induction. Each bar represents the mean percent CTL specific lysis  $\pm$  SEM from three replicate wells. \* $P < 0.01$  relative to no treatment; NS, not significant. (b) HEK-293 cells (grey) or HEK-293 transfectants expressing CD200R1 on the cell surface (white) were stained with a fluorescein isothiocyanate-labelled anti-CD200R1 antibody and analyzed by flow cytometry to confirm expression. (c) HEK293 cells expressing CD200R1 were incubated with the indicated concentrations of PEG-M49, PEG-M52, or PEG-cApt to monitor aptamer-induced phosphorylation of the CD200R1 cytoplasmic tail. The phosphorylated form of CD200R1 was detected by western blot using an antibody specific to phosphorylated-CD200R1 cytoplasmic tail. Supernatant from CD200-expressing cells was used as a positive control. Both PEG-M49 and PEG-M52 induce CD200R1 intracellular phosphorylation.

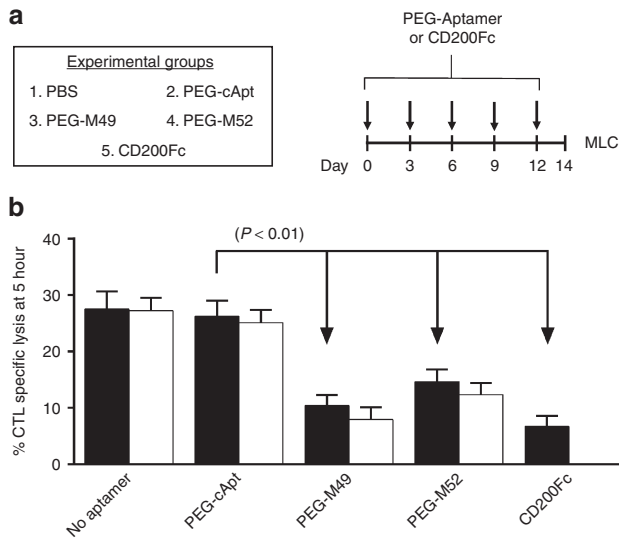
of CD200R1 agonistic aptamers could prolong survival of murine skin allografts.

To evaluate this possibility, BALB/c skin grafts were transplanted onto C57BL/6 mice ( $n = 6$ ; day 0). The aptamers PEG-M49, PEG-M52, PEG-cApt (650 pmol, based on nucleic acid content) or CD200Fc (325 pmol) were then administered intravenously into these mice every 3 days over a period of 15 days in combination with low-dose rapamycin (0.5 mg/kg, i.p) given every 48 hours (Figure 5a). Rapamycin at this dosage has been shown to have no effect on graft survival when administered alone (Supplementary Figure S3 and data not shown). Treatment with PEG-M49 and PEG-M52 protected against allograft rejection when compared to phosphate-buffered saline (PBS) or PEG-cApt groups (Figure 5b) ( $P < 0.05$ , Mann-Whitney  $U$ -test). Importantly, at the time of last injection (day 15), all control group animals had rejected their skin grafts while only 16–32% of mice receiving PEG-M49 or PEG-M52 respectively displayed signs of graft rejection (Figure 5b). Thus, the level of immunosuppression observed for these aptamers in this transplantation model was comparable to that of a bivalent form (CD200Fc) of the natural ligand for CD200R1. As described previously, conjugation of aptamers to PEG has been shown to increase their circulatory half-life and

therapeutic potency.<sup>27,29,30</sup> Consistently, PEGylated M49 prolonged allograft survival to greater extent than non-PEGylated M49 (Supplementary Figure S3).

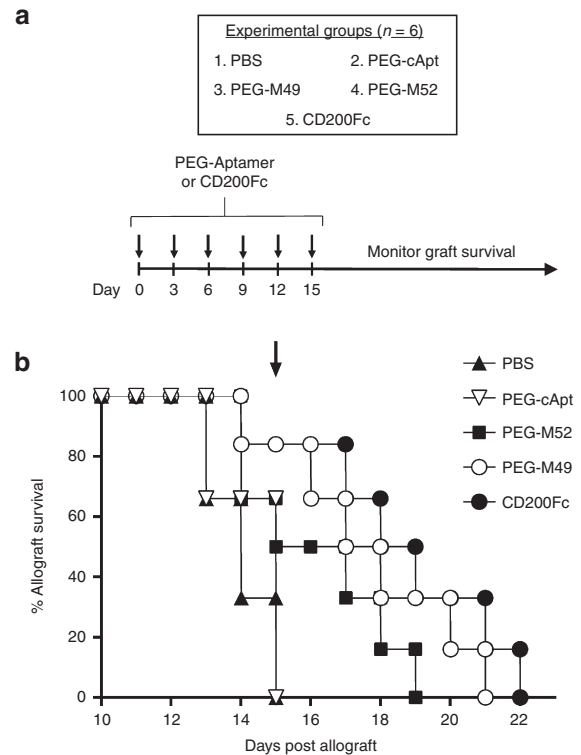
## Discussion

Ligands which bind to immune coreceptors and promote or impair signalling pathways have emerged as powerful immunotherapeutics. The best examples to date of such agents are neutralizing antibodies toward the coinhibitory molecules CTLA-4 and PD-1, which demonstrate profound activity in mobilizing immune responses and preventing tumor immune evasion in humans.<sup>40,41</sup> In contrast, agents which impart agonistic activity to such inhibitory coreceptors may prove useful in clinical situations where an immune response needs to be repressed such as transplantation, allergy, and autoimmunity. For instance, preclinical studies have shown that soluble forms of CD200 or a monoclonal antibody to CD200R1 can act as potent agonists to the CD200R1 inhibitory receptor in prolonging the survival of murine allografts,<sup>11,22</sup> reducing the severity of collagen-induced arthritis,<sup>7–9</sup> and in suppressing the progression of experimental autoimmune encephalomyelitis.<sup>24</sup> In the present study, we report the identification of short synthetic DNA aptamers that can mimic the functional (agonistic) properties of protein ligands such as CD200, in



**Figure 4** PEG-M49 and PEG-M52 function *in vivo*. (a) Experimental outline of *in vivo* experiment. BALB/c skin allografts (day 0) were transplanted onto C57BL/6 mice. These animals were subsequently treated over a 12-day period with tail vein injections of either 650 pmol PEG-M49, PEG-M52, PEG-cApt, or 325 pmol of CD200Fc at 72-hour intervals in combination with a low dose of rapamycin (0.5 mg/kg, 36 hours, i.p.). (b) After 14 days, mice were sacrificed and their splenocytes used as responder cells in *ex vivo* allo-MLC assays. Cultures were subsequently treated *in vitro* with further addition of aptamers or CD200Fc in culture (open bars) or left as is (closed bars). The *in vivo* administration of PEG-M49 and PEG-M52 was sufficient to suppress CTL induction without further addition of aptamer in culture ( $*P < 0.01$ ). Each bar represents the mean percent of CTL specific lysis  $\pm$  SEM from three replicate wells and data shown is representative of two independent experiments.

binding to CD200R1 and repressing immune cell responses. Two such aptamers, M49 and M52, were shown to potently suppress induction of CTL in allo-MLC assays (Figure 1). Importantly, PEGylated forms of M49 and M52 (PEG-M49 and PEG-M2) aptamers rapidly induced the phosphorylation of the CD200R1 cytoplasmic tail confirming that the aptamers work as agonists in inducing signalling in CD200R1-expressing cells in a similar manner to CD200. These two PEGylated aptamers retain immunosuppressive function in allo-MLC (Figure 3), their administration *in vivo* dampened immune activity in an *ex vivo* allo-MLC (Figure 4) and prolonged the survival of murine skin allografts (Figure 5). The treatment of mice with PEGylated aptamers or CD200Fc in transplantation experiments was halted at day 15 when all mice receiving either PBS or PEG-cApt had rejected their skin grafts. At that stage, allograft rejection was observed in only 16 or 32% of mice receiving PEGylated forms of M49 or M52 respectively, results that were similar to that of the bivalent CD200Fc (16%). Differences beyond this point may reflect different pharmacokinetic properties between PEGylated aptamers and recombinant protein rather than their ability to stimulate CD200R1 signalling. Notably, previous research has shown that continued CD200R1 stimulation using a transgenic mouse strain (CD200<sup>T9</sup>) which overexpresses CD200 upon doxycycline administration can induce full allograft acceptance, suggesting that sustained treatment with CD200R1 agonists such as PEGylated aptamers may



**Figure 5** PEG-M49 and PEG-M52 prolong the survival of transplanted murine skin grafts. (a) Experimental outline of *in vivo* experiment. C57BL/6 mice ( $n = 6$ ) received BALB/c skin allografts on day 0 and were treated every 3 days over 15 days with phosphate-buffered saline (PBS) (closed triangles) or 650 pmol of either PEG-cApt (open triangles), PEG-M49 (open circles), PEG-M52 (closed squares), or 325 pmol of CD200Fc (closed circles) in combination with low-dose rapamycin (0.5 mg/kg, 36 hours, i.p.). (b) Treatment with PEG-M49 and PEG-M52 significantly extended graft survival ( $P < 0.05$ , Mann-Whitney  $U$ -test) relative to control. Arrow represents time of last injection. Data shown is representative of two independent experiments.

similarly induce long-term allograft acceptance.<sup>13</sup> Furthermore, using the CD200<sup>T9</sup> model, it was shown that continued transgene expression was not necessary after allograft acceptance suggesting that ongoing or life-long administration of CD200R1 agonists may not be necessary.<sup>13</sup>

We have recently shown that functional DNA aptamers targeting TNF- $\alpha$ <sup>42</sup> and CEA,<sup>43</sup> do not trigger cytokine responses linked to innate immunity. Consistently, the PEGylated aptamers used in this study did not activate a TLR9 innate immune response. Furthermore, administration of DNA aptamers have not been associated with the generation of T and B cell responses.<sup>44,45</sup> These features are especially relevant to chronic or long-term administration of aptamers aimed at treating patients with allergies, arthritis, and autoimmune responses including rejection of tissues following transplantations. In contrast, most protein-based agents including humanized monoclonal antibodies do engender an immune response when administered chronically to patients.<sup>46</sup> Lastly, low production costs and scalable chemical synthesis make DNA aptamers especially viable agents for therapeutic applications.

Currently, there is one US Food and Drug Administration-approved aptamer called Macugen (Pegatinib), which is a

PEGylated protected RNA aptamer which binds to vascular endothelial growth factor to treat wet macular degeneration.<sup>47</sup> There are no immune modulatory aptamers in phase trials, however, several have been reported including aptamers targeting CTLA-4,<sup>31</sup> 4-1BB,<sup>32</sup> OX-40,<sup>33,34</sup> IL-6R,<sup>35</sup> IL-10R,<sup>36</sup> and CD28.<sup>37</sup> Interestingly, several agonistic aptamers developed to these receptors thus far have required dimerization through chemical scaffold or using a hybridized DNA technique.<sup>32–34,37</sup> In fact, the CD200Fc ligand used in the present study is actually bivalent in nature harbouring two CD200 domains, yet the monovalent PEGylated aptamers M49 and M52 have proven to be as effective in suppressing immune responses as CD200Fc.

In summary, we have shown as a proof of concept, that PEGylated aptamers directed at murine CD200R1 can act in an agonistic function to suppress immune response both *in vitro* and *in vivo*. Furthermore, these conjugated aptamers significantly prolong the survival of murine skin-allografts thereby highlighting their potential clinical utility. Further studies are ongoing to evaluate these aptamers in blocking acute and chronic inflammatory responses arising from other clinical situations including arthritis, trauma, and autoimmune conditions.

## Materials and methods

**Mice.** C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor ME). Mice were housed at five per cage in an accredited facility at the University Health Network, allowed food and water ad libitum, and used at 8–12 weeks of age.

**Aptamer selection by SELEX.** Single-stranded DNA aptamers (ssDNA) recognizing murine CD200R1 were identified using the polymerase chain reaction–based SELEX method.<sup>48,49</sup> Briefly, a 25 nucleotide long random synthetic oligonucleotide library flanked by 25-base long 5' and 3' primer regions (5'-GACGATAGCG GTGACGGCACAG ACG NNNNNNNNNNNNNNNNNNNNNNNNNNNCGTATGCC GCTTCCGTCGTCGTC-3') was synthesized (Integrated DNA Technologies, Coralville, IA) along with the corresponding primer sequences (forward: 5'-GACGATAGCGG TGACGGCACAGACG-3' and reverse: 5' GAGCGACGGAC GGAAGCGG CACACG-3'). A 4 nmol aliquot of the library representing  $\sim 2.5 \times 10^{15}$  sequences was adsorbed onto MagneHis Ni-Particles (Promega, Fitchburg, WI) at 37 °C for 1 hour to remove sequences which bound to the solid support. The resulting sublibrary was incubated for 1 hour at 37 °C with 10 µg of a recombinant HIS-tagged murine CD200R1 protein immobilized on MagneHis Ni-Particles suspended in 1 ml PBS (pH 7.4). Unbound and weakly bound sequences were removed by washing the beads with PBS for 5 minutes and protein–aptamer complexes were eluted with PBS containing 0.5 mol/l imidazole. Aptamers were recovered (Qiagen Nucleotide Removal kit) and the ssDNA pool enriched for murine CD200R1 ligands was amplified for the next round of selection using asymmetric polymerase chain reaction at a 10:1 forward:reverse primer ratio. Fifteen rounds of selection were performed with the selection stringency increasing as

the concentration of immobilized CD200R1 was reduced by a factor of 2 every three rounds while simultaneously increasing the number of wash steps. After the 15th cycle, selected DNA aptamers were cloned into pCR4-TOPO vector (Life Technologies, Burlington, Canada) and sequenced.

**Allogeneic mouse mixed lymphocyte culture (allo-MLC).** Agonistic CD200R1 aptamers were identified and evaluated for their ability to suppress the induction of cytotoxic T-lymphocytes (CTL) in 5-day allogeneic mouse mixed lymphocyte cultures (allo-MLC). Briefly,  $2.5 \times 10^5$  C57BL/6 responder splenocytes were incubated with an equal number of irradiated BALB/c stimulator cells in the presence of synthetic aptamers, PEGylated aptamers, or CD200Fc for 5 days. Levels of CTL induction were assayed by monitoring the release of <sup>51</sup>Cr from loaded P815 mastocytoma target cells over a 5-hour time period at a 25:1 effector-to-target ratio.

**Aptamer:CD200R1 nitrocellulose filter retention analysis.** The binding affinity of M49 and M52 toward CD200R1 was determined using a nitrocellulose filter binding assay. DNA aptamers were labelled at their 5' end with a terminal 32-P using T4 Polynucleotide Kinase (Life Technologies). Radiolabelled aptamers were purified using Micro Bio-Spin 6 desalting columns (BioRad, Mississauga, Canada) and adjusted to 5,000 CPM/µl in HEPES-buffered saline (20 mmol/l HEPES, 150 mmol/l NaCl, pH 7.4) with 1 mmol/l MgCl<sub>2</sub>. 5 µl aptamers were incubated with 15 µl of increasing concentrations of murine CD200R1 (Sino Biological, Beijing, China) in HEPES-buffered saline containing 1 mmol/l MgCl<sub>2</sub> and 0.01% BSA at 37 °C for 1 hour. After incubation, complexes were filtered through a dual filter system with an upper 0.4 µm nitrocellulose (BioRad) and lower nylon Hybond N<sup>+</sup> membrane (GE Healthcare, Mississauga, Canada) using a 96-well dot-blot apparatus. Membranes were exposed to film for 16 hours at room temperature, developed, and radiolabel signal quantified using ImageJ software (National Institute of Health, Bethesda, MD).

**PEGylation of DNA aptamers.** The 5' termini of aptamer M49, M52, and the control aptamer (cApt) were modified with a 20 kDa polyethylene glycol (PEG) moiety to increase their circulatory half-life. Briefly, a 5' amino group was incorporated into each DNA aptamer during their synthesis. A 100-molar excess of a mPEG-succinimidyl glutarate ester (Creative PEGWorks, Winston Salem, NC) was added stepwise over a period of 10 hours to 25 µmol/l solutions of the modified aptamers dissolved in 100 mmol/l NaHCO<sub>3</sub>/CH<sub>3</sub>CN (1:1 pH 8.5). The PEGylated aptamers were purified by ultrafiltration using Amicon Ultra Centrifugal Filters with a 30 kDa MWCO (Millipore, Billerica, MA) followed by size exclusion fast protein liquid chromatography using a Superdex 75 10/300 column (GE Healthcare) with 100 mmol/l NH<sub>4</sub>CO<sub>3</sub> as the eluent. Purified PEGylated-aptamer conjugates were lyophilized and resuspended in sterile PBS for subsequent experiments.

**Detection of CD200R1 phosphorylation.** Intracellular phosphorylation of CD200R1 in response to PEG-M49, PEG-M52, and PEG-cApt was detected using a rabbit polyclonal antibody specific to the phosphorylated cytoplasmic tail of CD200R1.<sup>50</sup> HEK-293 cells stably expressing murine

CD200R1 were serum-starved in OptiMEM (Life Technologies) medium for 3 hours and subsequently incubated for 30 minutes in OptiMEM medium containing either 2.5  $\mu\text{mol/l}$  PEG-M49, PEG-M52, PEG-cApt, or a CD200-positive cell lysate (positive control). Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (150 mmol/l NaCl, 1.0% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/l Tris, pH 8.0) containing 50 mmol/l NaF, 1 mmol/l  $\text{Na}_3\text{VO}_4$ , and protease inhibitors. Phosphorylated and unphosphorylated forms of CD200R1 were recovered by immunoprecipitation using an anti-CD200R1 (clone 2A10) monoclonal antibody (overnight 4 °C) and protein G agarose beads (Pierce, Rockford, IL). The phosphorylated form of CD200R1 was detected by western blot using the rabbit polyclonal antibody (1:1,000 dilution) and anti-rabbit horseradish peroxidase (1:15,000 dilution).

**Activity of PEGylated aptamers in vivo.** C57BL/6 mice received BALB/c skin allografts followed by tail vein injections at day 0, 3, 6, 9, and 12 of either PEG-M49, PEG-M52, PEG-cApt, (650 pmol, based on oligonucleotide content) or CD200Fc (325 pmol) dissolved in 0.2 ml PBS, pH 7.4 in combination with low-dose (0.5 mg/kg) rapamycin administered intraperitoneally every 48 hours. On day 14, mice were sacrificed and their splenocytes used as responder cells in 5-day *ex vivo* allo-MLC with or without further aptamer addition *in vitro*.

**Analysis of aptamer-induced innate immune responses.** PBS, PEGylated aptamers (650 pmol, based on oligonucleotide content) or a TLR9 ligand CpG ODN (ODN 1826, Invivogen, CA) were administered by tail vein injection to naive C57BL/6 mice ( $n = 3$ ). Mice were sacrificed 6 hours later, and the serum levels of TNF $\alpha$  or IL-6 quantified using commercial ELISA sets (R&D Systems, Minneapolis, MN).

**Allogeneic skin graft transplantation.** PEG-M49 and PEG-M52 were evaluated for their ability to prolong survival of allogeneic murine skin grafts. C57BL/6 mice ( $n = 6$ ) received BALB/c skin allografts prior to receiving six tail vein injections of either PEG-M49, PEG-M52, PEG-cApt (650 pmol), or CD200Fc (325 nmol) dissolved in 0.2 ml PBS, pH 7.4 once every 3 days over 15 days in combination with low-dose (0.5 mg/kg) rapamycin administered intraperitoneally every 48 hours. Graft survival was monitored daily.

**Statistical analysis.** *P* values for survival analysis were determined using a Mann–Whitney *U*-test while *P* values for MLC assays were calculated using a Student's *t*-test.

### Supplementary material

**Figure S1.** Modification and purification of DNA aptamers with a 20kDa PEG.

**Figure S2.** PEGylated aptamers do not activate a TLR9 Innate Immune Response.

**Figure S3.** PEGylation is necessary for prolongation of allograft survival.

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