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ImmunoPET imaging of murine CD4⁺ T cells using anti-CD4 cys-diabody: Effects of protein dose on T cell function and imaging

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Abstract

PURPOSE—Molecular imaging of CD4⁺ T cells throughout the body has implications for monitoring autoimmune disease and immunotherapy of cancer. Given the key role of these cells in regulating immunity, it is important to develop a biologically inert probe. GK1.5 cys-diabody (cDb), a previously developed anti-mouse CD4 antibody fragment, was tested at different doses to assess its effects on positron emission tomography (PET) imaging and CD4⁺ T cell viability, proliferation, CD4 expression, and function.

PROCEDURES—The effect of protein dose on image contrast (lymphoid tissue-to-muscle ratio) was assessed by administering different amounts of ⁸⁹Zr-labeled GK1.5 cDb to mice followed by PET imaging and *ex vivo* biodistribution analysis. To assess impact of GK1.5 cDb on T cell biology, GK1.5 cDb was incubated with T cells *in vitro* or administered intravenously to C57BL/6 mice at multiple protein doses. CD4 expression and T cell proliferation were analyzed with flow cytometry and cytokines were assayed.

RESULTS—For immunoPET imaging, the lowest protein dose of 2 µg ⁸⁹Zr-labeled GK1.5 cDb resulted in significantly higher % injected dose/gram in inguinal lymph nodes (ILN) and spleen compared to the 12 µg protein dose. *In vivo* administration of GK1.5 cDb at the high dose of 40 µg caused a transient decrease in CD4 expression in spleen, blood, lymph nodes, and thymus, which recovered within 3 days post-injection; this effect was reduced, although not abrogated, when 2 µg was administered. Proliferation was inhibited *in vivo* in ILN but not the spleen by injection of 40 µg GK1.5 cDb. Concentrations of GK1.5 cDb in excess of 25 nM significantly inhibited CD4⁺ T cell proliferation and interferon-γ production *in vitro*. Overall, using low dose GK1.5 cDb minimized biological effects on CD4⁺ T cells.

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Conflict of Interest Statement

A. M. W. is a stockholder in and consultant to ImaginAb, Inc.

CONCLUSIONS—Low dose GK1.5 cDb yields high-contrast immunoPET images with minimal effects on T cell biology *in vitro* and *in vivo*, and may be a useful tool for investigating CD4⁺ T cells in the context of preclinical disease models. Future approaches to minimizing biological effects may include the creation of monovalent fragments or selecting anti-CD4 antibodies which target alternative epitopes.

Keywords

immunoPET; CD4; lymphocytes; T cells; T cell function; antibody engineering; diabody; zirconium-89; positron emission tomography

Introduction

CD4⁺ T cells orchestrate the immune system, guiding both pro- and anti-inflammatory responses. The CD4 co-receptor interacts with the T cell receptor (TCR) complex and the major histocompatibility complex class II (MHC II), which is expressed on antigen presenting cells, to increase CD4⁺ T cell sensitivity to antigen. Upon antigen recognition, CD4⁺ T cells become activated, proliferate, produce cytokines for immune cell recruitment and activation, and assist B cells to produce antibodies and activate other immune cells [1]. As a consequence of their role in regulating immunity, accurate measurement of CD4⁺ T cells has implications for monitoring and treatment of a variety of diseases and disorders, including HIV infection [2, 3], autoimmune diseases such as rheumatoid arthritis [4, 5] and inflammatory bowel disease [6], and cancer [7]. Current methods of collecting CD4⁺ T cells include peripheral blood draws, which are relatively noninvasive, and biopsies, which allow testing from a precise location of interest. However, these methods give a limited representation of tissue-resident CD4⁺ T cells throughout the immune system [8]. In humans only ~2% of total lymphocytes are in the blood at any time; the majority reside in lymphoid tissues (e.g. spleen and lymph nodes) as well as in non-lymphoid organs [9, 10]. Biopsies can sample solid tissues, but are invasive and can give variable results due to CD4⁺ T cell inhomogeneity. Furthermore, growing interest in tumor-infiltrating lymphocytes and the influence of CD4⁺ T cells on the outcome of cancer immunotherapy [7] suggests a need for measurement of T cells throughout the entire tumor and nearby lymphoid organs [11]. For a comprehensive assessment of CD4⁺ T cells, a method that can simultaneously survey CD4⁺ T cell reservoirs throughout the body is desirable.

Radionuclide-based molecular imaging, including positron emission tomography (PET) and single photon emission computed tomography (SPECT), enables sensitive, noninvasive, whole-body imaging of cells, biomarkers, and cellular processes. Sites of inflammation can be identified by adoptive transfer of *ex vivo* radiolabeled autologous lymphocytes and tracking their migration after reinfusion. This is done clinically using ¹¹¹In-oxine or ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}TcHMPAO) to track cells with SPECT [8]; for tracking lymphocytes using PET, ⁸⁹Zr- and ⁶⁴Cu-labeled probes are emerging as effective candidates [12, 13]. To image an endogenous subset of cells, the targeting of a biomarker-specific probe is required. Antibody-based imaging (immunoPET or immunoSPECT) combines the exquisite specificity of antibodies and the sensitivity and tissue penetration of nuclear imaging to noninvasively image and quantitate endogenous cell surface biomarkers.

Radiolabeled antibodies have been used to image CD4⁺ T cells, mainly in preclinical settings. Rubin et al. utilized ¹¹¹In-labeled GK1.5 anti-CD4 antibody to assess distribution of murine CD4⁺ T cells with gamma camera imaging [14]. In a murine model of colitis, ¹¹¹In-labeled YTS 177 non-depleting anti-CD4 antibody was used for SPECT imaging of excess CD4⁺ T cells in the gut [6]. ImmunoSPECT with ¹¹¹In-labeled anti-CD4 antibody enabled tracking of CD4⁺ T cells in simian-HIV-infected rhesus macaques, and *ex vivo* biodistribution data was used to revise the proposed number of total lymphocytes in the human body [2]. Clinical use of anti-CD4 immunoSPECT has been explored in the context of rheumatoid arthritis with mixed results. Uptake in inflamed joints correlated well with clinical symptoms in one study, which utilized intact ^{99m}Tc-labeled anti-CD4 antibody Max. 16H5 [5], but in a later study, a ^{99m}Tc-labeled Fab fragment of the same antibody identified only 68% of clinically affected joints [4]. These results led the authors to suggest that the presence of CD4⁺ T cells does not always correlate with pain and swelling in arthritic joints.

A major concern in the development of new PET tracers is the effect on target cells. Ideally, a tracer should have minimal effects on cell viability and function. Intact antibodies mediate effector function via the Fc region and can induce depletion of or functional changes in cells expressing the target antigen. For example, intact rat anti-mouse CD4 antibody GK1.5 depletes CD4⁺ T cells *in vivo* and can affect induction of proliferation and cytokine release [15–17]. In addition, intact antibodies have a long half-life (serum $t_{1/2}$ = 1–3 weeks) due to recycling through the neonatal Fc receptor, and require several days of clearance to acquire a high-contrast image. To address the drawbacks of Fc-mediated effector functions and long half-life, antibodies can be engineered into various fragments with customized pharmacokinetics, conjugation capabilities, Fc receptor binding ability, and excretion route [18].

We previously developed an anti-CD4 antibody fragment, GK1.5 cys-diabody (cDb), for immunoPET imaging of murine CD4⁺ T cells and described its use in monitoring CD4⁺ T cell reconstitution after hematopoietic stem cell transplantation [19]. GK1.5 cDb lacks the Fc region and clears rapidly through the kidney, enabling same- or next-day imaging. Subsequent studies demonstrated that GK1.5 cDb caused decreased surface expression of CD4, which prompted investigation of the potential impact of GK1.5 cDb on CD4⁺ T cells. Here, the effects of GK1.5 cDb dose on CD4⁺ T cell biology and immunoPET imaging were explored. A series of protein doses was evaluated for changes on T cell surface CD4 expression, antigen-driven proliferation, cytokine production, immunoPET image contrast, and biodistribution.

Materials and Methods

Animals

Female C57BL/6 and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice between 6–12 weeks of age were obtained from Jackson Laboratories and housed by the Department of Laboratory Animal Medicine at the University of California, Los Angeles (UCLA). Animal studies were conducted under protocols approved by the Chancellor's Animal Research Committee at UCLA. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Production, conjugation, and labeling of GK1.5 cDb

Production, purification, conjugation, and radiolabeling of GK1.5 cDb were performed as previously described [19]. Briefly, GK1.5 cDb was reduced with 20-fold molar excess of tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Pierce) and site-specifically conjugated with 20-fold molar excess of maleimide-desferrioxamine (malDFO; Macrocyclics). MalDFO-GK1.5 cDb (272 µg) was radiolabeled with Zr-89 (40.3 MBq; 3D Imaging), purified, and labeling efficiency and radiochemical purity were assessed with instant thin layer chromatography. For conjugation to maleimide-biotin (malBiotin; Sigma), the procedure was identical to the conjugation to malDFO except a 20-fold molar excess of malBiotin was used.

Production of soluble murine CD4

Polymerase chain reaction (PCR) was used to amplify DNA encoding the extracellular domain of murine CD4 (Lys1 – Val360) from the complete mouse CD4 sequence (Sino Biologicals). 5' *AgeI* and 3' *NotI* restriction digest sites (underlined) were added during amplification using the following primers: Forward primer: 5' – CACACAACCGGTAAGACGCTGGTGCTGGGGAA; reverse primer: 5' – TGTGTGCGCGGCCGCAACCTGGATCCTGGAGTC. The PCR product was digested with *AgeI* and *NotI* (New England Biolabs) and inserted into the TOPO subcloning vector which previously contained the 2.43 minibody [20], to add a 5' mouse Ig Kappa secretion signal sequence and a 3' hexahistidine tag. *XbaI* and *EcoRI* (New England Biolabs) were then used to subclone the sCD4-His into the mammalian expression vector pEE12 (Lonza). Sequence verification was performed at the GenoSeq core at UCLA. NS0 electroporation, clonal selection using Ni-NTA HisSorb plates (Qiagen), protein production, and Ni-NTA purification were performed using previously described methods [20] except anti-CD4 (clone GK1.5; UC San Francisco Monoclonal Antibody Core) and alkaline phosphatase-conjugated anti-rat Fc (Sigma) were used to detect sCD4-His on the HisSorb plates. Concentrations of purified sCD4 or GK1.5 cDb were determined by measuring UV absorbance at 280 nm with a NanoDrop spectrophotometer (Thermo) and using the calculated molecular weight and the theoretical extinction coefficient (Geneious).

Soluble antigen binding assays

For the enzyme-linked immunosorbent assay (ELISA), 96-well high-binding polystyrene plates (Costar) were coated overnight at 4°C with 100 µl of 5 µg/ml soluble murine CD4. The next day, plates were washed and blocked with 2% non-fat milk (Bio-Rad) in PBS for 2 hours at room temperature. GK1.5 cDb-malBiotin or biotinylated GK1.5 Ab (Biolegend) were serially diluted (1:4 dilutions; 500 nM-0.0085 nM, in triplicate) in 2% non-fat milk and added to the plate for a 1 hour incubation. After washing with 0.1% PBS-Tween, streptavidin-alkaline phosphatase (Jackson ImmunoResearch) was added at a dilution of 1:2500 for 15 minutes, washed, and detected using *p*-nitrophenyl phosphate substrate (Sigma). Absorbance at 405 nm was read using an Infinite m200 Pro plate reader (Tecan). Affinities were estimated using a one site – specific binding model in Prism (GraphPad).

To investigate complex formation, soluble murine CD4 and GK1.5 cDb were incubated at a mass ratio of 2:1 at room temperature for 10 minutes and chromatographed on a Superdex 75 (GE) size exclusion chromatography (SEC) column using PBS for running buffer.

ImmunoPET imaging and biodistribution

MicroPET and microCT imaging was performed as described previously [19]. A range of ^{89}Zr malDFO-GK1.5 cDb doses (2–40 μg ; 0.26–5.2 MBq) were prepared in 100 μl saline and injected intravenously into mice. Mice were anesthetized with 2% isoflurane and PET and CT images were acquired 20 h post-injection. MicroPET images were acquired on an Inveon PET (Siemens) scanner for 600 sec and reconstructed using a 3D-ordered subset expectation maximization (OSEM) algorithm with 2 iterations, followed by maximum *a posteriori* (MAP) with 18 iterations (beta=0.1) with a zoom factor of 2.1, without attenuation correction. Whole-body CT images were acquired using the MicroCAT II (Imtek) scanner, with the x-ray source based at 70 kVp and 500 μA and an exposure time of 180 s (0.5 s per projection). A Feldkamp reconstruction algorithm was applied [21]. A Medical Imaging Data Examiner (AMIDE) software (<http://amide.sourceforge.net>) was used to view PET and CT scans. Images are displayed as 25 mm (coronal) or 2 mm (transverse) maximum intensity projections (MIP). Following the final PET/CT scan, *ex vivo* biodistribution was performed. Organs were removed, weighed, and counted in a Wizard 3" automatic gamma counter (Perkin Elmer) and the decay-corrected percent injected dose/gram (% ID/g) for each organ was calculated.

Flow cytometry

Blood from euthanized C57BL/6 mice was collected in a heparinized tube. Thymus, lymph nodes, and spleen were harvested and single-cell suspensions prepared by mashing organs through 70 μm nylon filters using a syringe plunger. Filters were rinsed with PBS/2% FBS and red blood cells were lysed with RBC Lysis Buffer (Biolegend). Cells were then incubated in PBS/2% FBS for 15 minutes on ice, and stained for 30 min on ice with the following fluorescently labeled antibodies: anti-CD45-PE/Dazzle (clone 30F11), anti-CD4-PE (RM4-4), anti-CD4-BV421 (GK1.5), anti-CD8-AlexaFluor 700 (53.67) (Biolegend), and anti-CD3-APC (17A2; eBioscience). Stained cells were washed twice and analyzed on a BD Fortessa SORP X-20 flow cytometer. Blood and organs from individual mice were processed and stained as single samples. Data were analyzed using FlowJo analysis software (FlowJo LLC).

For analysis of changes in CD4 expression, C57BL/6 mice were injected with 2 or 40 μg GK1.5 cDb ($n=3-4$ mice/group). After 24 or 72 hours, blood, spleen, inguinal lymph nodes, and thymus were processed as above and the mean fluorescence intensity (MFI) of CD4 (BV421 or PE) by CD45⁺ cells was calculated for each organ. The average MFI for each organ in the untreated control group was designated as 100% (maximum), and individual values from experimental mice were compared to the maximum to evaluate modulation of CD4 expression. Change in expression is reported as mean $\pm$ standard deviation per experimental group of mice.

Proliferation assays

CD4⁺ T cells were isolated from the spleen and inguinal lymph nodes of C57BL/6 or OT-II mice. Organs were harvested, mashed through a mesh filter and washed with PBS/2% FBS. Negative selection was performed using the EasySep™ Mouse CD4⁺ T Cell Isolation Kit and EasySep magnet (StemCell Technologies). Purity was ~93% as determined by flow cytometry. Cells were labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) according to a published protocol [22].

To assay antigen-specific T cell proliferation, wild type C57BL/6 splenocytes were plated in 96-well plates (2×10^5 cells/well, in triplicate) and incubated overnight in the presence of 1 mg/ml ovalbumin (OVA) (Sigma). 24 hours later, 1×10^5 CFSE-labeled OT-II CD4⁺ T cells were added to the splenocytes, followed immediately by addition of various concentrations (0–100 nM, in triplicate) of GK1.5 cDb. Proliferation was assayed 72 hours later using flow cytometry. The proliferation platform of FlowJo was used to calculate percent divided (the percent of CFSE-labeled cells that divided at least once) and replication index (the fold-expansion of divided cells).

To assay antigen-specific T cell proliferation *in vivo*, 2×10^6 CFSE-labeled OT-II CD4⁺ T cells were injected intravenously into C57BL/6 recipients. 24 hours later, mice were intravenously administered PBS, 100 μ g OVA, 100 μ g OVA + 2 μ g GK1.5 cDb, or 100 μ g OVA + 40 μ g GK1.5 cDb. After 72 hours, the spleen, ILN, thymus, and blood were harvested, processed, and analyzed with flow cytometry to determine extent of proliferation.

Cytokine assays

Cytokine analysis was performed by the Immune Assessment Core at UCLA, using the ProcartaPlex Mouse Essential Th1/Th2 Cytokine Panel (6 plex) (eBioscience). Briefly, 50 μ L undiluted supernatant samples from the *in vitro* CD4⁺ T cell proliferation assays (in duplicate) were mixed with 50 μ L magnetic beads and incubated overnight at 4°C, with shaking for 30 minutes before and after the incubation period. Plates were then washed three times manually with manufacturer-supplied wash buffer. 25 μ L of biotinylated detection antibody was added and incubated for 30 min at room temperature. After three washes, 50 μ L streptavidin-phycoerythrin conjugate was added to the reaction mixture and incubated for 30 minutes at room temperature. Following an additional three washes, beads were resuspended in manufacturer-supplied reading buffer and shaken for 5 min at room temperature. Fluorescence was quantified using a Luminex FLEXMAP 3D instrument. Data were analyzed using MILLIPLEX Analyst 5.1 software.

Statistical analysis

Summary statistics (means, standard deviations, and standard errors) were computed for each group. Two-sample Student's t-tests were used to compare the means of outcome measurement between groups. To correct for multiple comparisons in evaluating multiple organs and dosages in the *ex vivo* biodistribution study and the studies involving *in vivo* administration of GK1.5 cDb, the false discovery rates in the Benjamini-Hochberg step-up procedure [23] have been reported with a 5% threshold for significance.

Unless indicated otherwise, values are reported as mean $\pm$ standard deviation. For flow cytometry assays, cytokine assays, and imaging experiments, experimental groups were compared individually to control groups. For the GK1.5 cDb dose escalation imaging and *ex vivo* biodistribution studies, the 2, 6, and 40 μ g protein doses were compared to the previously published 12 μ g protein dose [18]. Analyses of experimental data were performed using Prism (GraphPad; version 7) and SAS (SAS Institute; version 9.4).

Results

GK1.5 cDb retains specificity and affinity for murine CD4

GK1.5 cDb is a covalent dimer of two single-chain variable fragments (Fig 1a). To confirm that GK1.5 cDb binds to CD4, antibody-antigen complex formation in solution was assayed by SEC. Free sCD4 (50 kDa) and free GK1.5 cDb (52 kDa) eluted as separate peaks (Fig 1b); after incubation of sCD4 and GK1.5 cDb at a 2:1 molar ratio, a major peak representing a higher molecular weight complex eluted (Fig 1b). To evaluate the affinity for GK1.5 cDb to CD4, a saturation binding assay was performed using parental GK1.5 Ab as a control. The apparent affinities for both were similar (GK1.5 Ab $K_d = 1.1 \pm 0.06$ nM; GK1.5 cDb $K_d = 2.7 \pm 0.20$ nM; $n = 3$ independent experiments) (Fig 1c).

ImmunoPET with low-dose ^{89}Zr -malDFO-GK1.5 cDb yields high-contrast images of lymphoid organs

MalDFO-GK1.5 cDb was successfully radiolabeled with Zr-89. Radiolabeling efficiency and radiochemical purity of ^{89}Zr -malDFO-GK1.5 cDb as determined by instant thin layer chromatography were both $\sim 99\%$. Specific activity of ^{89}Zr -malDFO-GK1.5 cDb was 0.12 MBq/ μ g. To determine the effect of protein dose on specific uptake by lymphoid organs, 2, 6, 12, or 40 μ g (0.26–5.0 MBq) of ^{89}Zr -malDFO-GK1.5 cDb were injected intravenously into C57BL/6 mice ($n=4$ for 2, 6, and 40 μ g protein doses; $n=3$ for 12 μ g protein dose, previously published [19]). PET images at 20 h post-injection (p.i.) showed uptake of ^{89}Zr -malDFO-GK1.5 cDb in spleen and cervical, axillary, inguinal, and popliteal lymph nodes at all protein doses (Fig 2). Consistent with previously published studies [19], thymic uptake of ^{89}Zr -malDFO-GK1.5 cDb was not observed on microPET images; however, small regions of uptake are visible at the anterior end of the lung cavity and may represent parathymic lymph nodes which are normally associated with the thymus [24]. Higher protein doses resulted in a blocking effect, as seen by reduced contrast of lymphoid organs in the 12 and 40 μ g images (Fig 2).

Ex vivo biodistribution studies confirmed that uptake in lymph nodes and spleen was high. The %ID/g was significantly higher at the 2 μ g dose compared to the previously published 12 μ g dose [19] in ILN (2 μ g: 106 ± 13 ; 12 μ g: 45 ± 5.3 %ID/g; $p<0.05$). Both the 2 μ g and 6 μ g doses resulted in significantly higher uptake in the spleen compared to the 12 μ g dose (2 μ g: 70 ± 1.4 ; 6 μ g: 46 ± 5.0 ; 12 μ g: 30 ± 3.4 %ID/g; $p<0.05$) (Fig 3a; Table 1). Uptake in lymphoid organs was not significantly different between the 40 and 12 μ g doses. Thymic uptake was not observed at any dose (Fig 3a; Table 1). Lymphoid tissue:muscle ratios were also higher at lower protein doses (Fig 3b). Non-specific background uptake was observed in

organs of clearance, primarily the kidney as GK1.5 cDb is below the molecular weight threshold (~60 kDa) for renal clearance (Fig. 3a; Table 1).

GK1.5 cDb causes transient decrease in CD4 expression in vivo

To investigate effects of GK1.5 cDb on the expression of CD4 by CD4⁺ T cells, we injected 40 µg GK1.5 cDb into C57BL/6 mice and measured surface CD4 expression by T cells in blood, spleen, ILN, and thymus after one or three days. Due to the possibility that GK1.5 cDb could induce downmodulation of CD4, or block staining by fluorescently labeled GK1.5 antibody, flow studies on CD4 expression were performed using both GK1.5 and RM4-4 anti-CD4 antibodies, which have distinct, non-overlapping epitopes on extracellular CD4 [25]. CD4 expression (measured with RM4-4) was significantly decreased one day p.i. in mice treated with GK1.5 cDb compared to control untreated mice by 63 ± 5% (blood; p<0.005), 64 ± 7% (spleen; p<0.005), 53 ± 9% (ILN; p<0.05), and 33 ± 10% (thymus; p<0.05) (Fig 4a). Three days later, CD4 expression on T cells had recovered in blood, LN, and thymus. Splenic CD4⁺ T cells showed a slower recovery on day 3 (21 ± 8% decrease in MFI), but the difference was not statistically significant. Despite the decrease in expression as measured by mean fluorescence intensity, there were no significant changes in the percentages of CD45⁺CD4⁺ or total CD3⁺ T cells, showing that CD4⁺ T cells were not depleted by administration of GK1.5 cDb (Fig 4b-c). Staining with GK1.5 Ab confirmed the results of staining with RM4-4, indicating that the epitopes for both antibodies were accessible (Fig 4d-e). The lesser extent of decreased CD4 expression by T cells in the thymus suggests that GK1.5 cDb is less able to interact with CD4⁺ T cells in the thymus compared to other lymphoid organs. This parallels the results of imaging and biodistribution studies, which showed no specific uptake of ⁸⁹Zr-malDFO-GK1.5 cDb in the thymus (Fig 2a, Fig 3a, Table 1) [19].

GK1.5 cDb alters CD4⁺ T cell proliferation and cytokine release in a dose-dependent manner *in vitro*

GK1.5 cDb could potentially affect antigen-specific CD4⁺ T cell proliferation because it binds to an epitope on Domain 1 of CD4 [26] which interacts with the β2-domain of MHC II on antigen presenting cells [27]. To evaluate this effect *in vitro*, OT-II CD4⁺ T cells (which recognize peptides 329–339 of OVA presented by MHC II) were cultured with OVA-pulsed splenocytes and serial concentrations of GK1.5 cDb. At 25 nM and above, GK1.5 cDb significantly inhibited the percentage of OT-II CD4⁺ T cells able to initiate cell division (Fig 5a). These concentrations also inhibited cells that were able to initiate division from continuing to proliferate (Fig 5b). Lower concentrations of GK1.5 cDb did not show any inhibition of proliferation; interestingly, the lowest concentration (0.097 nM) seemed to stimulate proliferation, although this effect was not significant (p=0.064).

An additional readout of T cell activation is cytokine production. Changes in interferon-γ (IFNγ) production by cells *in vitro* paralleled the dose-dependent GK1.5 cDb-induced inhibition of proliferation. The concentration of IFNγ was significantly decreased at 25 nM and 100 nM GK1.5 cDb (Figure 5c). Levels of other cytokines were also assayed, but no consistent trends were seen (data not shown).

GK1.5 cDb decreases CD4 expression and inhibits CD4⁺ T cell proliferation in a dose-dependent manner *in vivo*

To determine the impact of protein dose on surface CD4 expression by T cells *in vivo*, GK1.5 cDb was administered to mice at the 2 and 40 µg doses used in the imaging study. The 2 µg dose of GK1.5 cDb resulted in decreased expression of surface CD4 one day p.i., but to a lesser extent than the 40 µg dose (Fig 6a). To expand on the *in vitro* proliferation studies, C57BL/6 mice were administered OT-II CD4⁺ T cells one day prior to the injection of OVA and the low or high dose of GK1.5 cDb. Effects of GK1.5 cDb on antigen-specific proliferation *in vivo* were organ- and dose-dependent. Proliferation of OT-II CD4⁺ T cells in spleen was not significantly inhibited after administration of either 2 µg or 40 µg of GK1.5 cDb (Fig 6b-c). OT-II CD4⁺ T cells isolated from the ILN showed significantly inhibited proliferation at 40 µg, but not at 2 µg, confirming that higher doses of GK1.5 cDb result in impaired CD4⁺ T cell antigen-driven division (Fig 6b-c).

Discussion

The CD4 co-receptor is required for efficient activation of CD4⁺ T cells by antigen presenting cells, and modulation of co-receptor availability by an immunoPET imaging probe could affect the ability of cells to respond to antigen presentation. Investigation of the potential impact of GK1.5 cDb on CD4⁺ T cells is of particular importance because the parental intact GK1.5 Ab has long been used for its CD4⁺ T cell depleting properties; furthermore, GK1.5 binds to the domain of CD4 that interacts with MHC II [26]. Its ability to suppress the induction of antigen-specific CD4⁺ T cell proliferation and cytokine release has been documented [16], and does not require the Fc region, as demonstrated by a study showing that the monovalent Fab fragment of GK1.5 inhibits induction of CD4⁺ T cell proliferation in response to antigen *in vitro* [17]. In addition, a bivalent F(ab')₂ GK1.5 fragment inhibited humoral immunity in mice [28], indicating that fragments of GK1.5 can have potentially severe effects on not only CD4⁺ T cells but indirectly on other compartments of the immune system as well. Here, various protein doses relevant to those used for imaging were assessed to determine whether GK1.5 cDb modulated CD4⁺ T cell viability, surface CD4 expression, proliferation, or cytokine production.

Anti-CD4 immunoPET provides a method of noninvasively monitoring CD4⁺ T cells in a comprehensive manner, and can complement and expand on the information gained from blood samples and biopsies. *Ex vivo* biodistribution data collected from the dose-escalation imaging study showed that the 2 µg protein dose of ⁸⁹Zr-malDFO-GK1.5 cDb resulted in significantly higher %ID/g in spleen and ILN compared to protein doses of 12 or 40 µg (Figure 3a; Table 1). These results are favorable, as the 2 µg protein dose resulted in a milder effect on decreased CD4 expression, and did not inhibit CD4⁺ T cell proliferation *in vivo*. Lack of specific ⁸⁹Zr-malDFO-GK1.5 uptake in the thymus was consistent at all protein doses. We considered that thymic uptake might be Fc-region dependent [19], since the parental intact GK1.5 Ab depletes CD4⁺ T cells in the thymus successfully; however, Rubin et al. imaged with intact GK1.5 and also saw no specific thymic uptake [14]. Interestingly, thymic T cells showed a small, but significant, decrease in CD4 expression after *in vivo* administration of GK1.5 cDb, suggesting that a small amount of GK1.5 cDb is able to enter

the thymus (Fig 4a, 4d). Parathymic LN were likely isolated with the thymus and may have acted as a source of extrathymic CD4⁺ T cells, which could have affected the results of biodistribution analysis and CD4 expression studies. Delivery to, retention, and activity of anti-CD4 Abs and Ab fragments in the thymus warrants further investigation.

The effect of GK1.5 cDb on CD4 expression was dose-dependent, as mice that received 2 µg GK1.5 cDb retained higher surface CD4 expression on CD45⁺ cells from the spleen, blood, ILN, and thymus than mice that received the 40 µg dose (Fig 6a). Although the decrease in expression was significant, CD4 was still detectable at both doses. The change in CD4 MFI was transient and expression levels recovered within three days post-injection (Fig 4a, 4d). Although cell surface expression of CD4 was decreased, the percentages of total CD3⁺ T cells and CD45⁺CD4⁺ cells in the spleen, blood, ILN, and thymus were not altered after one or three days, indicating that GK1.5 cDb does not deplete CD4⁺ T cells *in vivo* (Fig 4b-c, 4e). Reduced CD4 expression levels are assumed to be due to GK1.5 cDb-induced downmodulation of CD4.

In vivo administration of GK1.5 cDb demonstrated dose-dependent effects on the proliferation of adoptively transferred CFSE-labeled OT-II CD4⁺ T cells: 2 µg GK1.5 cDb did not significantly inhibit OVA-induced proliferation in the spleen or ILN, but 40 µg did inhibit proliferation in the ILN (Fig 6b-c). *In vitro* functional assays demonstrated a similar result; only at concentrations of 25 nM and above were cell division and IFNγ release inhibited (Fig 5). However, the lowest dose of 0.097 nM caused increased proliferation and presents an interesting possibility: GK1.5 cDb administered at very low doses could have an immune-stimulating effect. Furthermore, the decreased proliferation seen at higher doses both *in vitro* and *in vivo* suggests that GK1.5 cDb could be used at higher doses to suppress CD4⁺ T cell proliferation without depleting the cells. Additional studies would be required to determine the optimal dose for agonistic or antagonistic effects of GK1.5 cDb activity *in vivo*.

Alternative approaches to reduce probe biological activity include creating a monovalent antibody fragment to prevent crosslinking and internalization of the biomarker, or targeting an epitope that is less likely to be involved in critical interactions such as CD4-MHC II binding. Additionally, reduction of radiation dose is especially important when imaging CD4⁺ T cells as they are particularly radiosensitive [29]. Here, the sensitivity of PET imaging enabled levels of administered activity as low as 0.26 MBq. Radiation produced by the microCT is also of concern, as ionizing radiation can induce multiple effects in lymphoid populations including changes in gene and protein expression, stimulation or inhibition of proliferation, and apoptosis [29–31]. To limit exposure, the use of advanced microCT instruments which require lower radiation doses would be beneficial.

While this work demonstrates the utility of anti-CD4 cDb in imaging CD4⁺ T cells throughout an organism, one disadvantage is that subsets of CD4⁺ T cells and other CD4-expressing cells such as dendritic cells cannot be distinguished using a single anti-CD4 probe. Therefore, GK1.5 cDb is limited in its ability to identify, for example, whether signal is due to a pro-inflammatory Th1 cell, an inflammation-suppressing Treg cell, or any another CD4⁺ cell. More specific biomarkers would need to be assessed either alone or in tandem

with GK1.5 cDb in order to localize and quantify certain CD4⁺ cell populations. Although GK1.5 cDb alone cannot provide information about the various subsets present, its use in imaging total CD4⁺ T cells is still highly valuable; anti-CD4 immunoPET gives spatial and quantifiable data about CD4⁺ T cells throughout the entire body in contrast to blood samples and biopsies, which sample a limited proportion of total CD4⁺ T cells. GK1.5 cDb can be used to investigate CD4⁺ T cells in mouse models of disease and to test treatment efficacy rapidly and noninvasively. The ability to image and quantify total CD4⁺ T cells has implications for monitoring diseases including HIV infection [2, 3], colitis [6], and rheumatoid arthritis [4, 5]. Recent interest in the potential of CD4⁺ T cells for tumor immunotherapy also suggests a role for careful monitoring of CD4⁺ T cells in the context of anti-tumor immunity [7]. Further studies may include use of this probe to study cell trafficking throughout the immune system and recruitment of CD4⁺ T cells to sites of inflammation.

Conclusions

Low dose GK1.5 cDb yields high-contrast immunoPET images with minimal biological effects on CD4⁺ T cells, and is a promising agent for noninvasive, whole-body investigation of CD4⁺ T cells in mice. When developing new imaging probes, dose may therefore be a key condition for optimization in order to image a target without perturbing the biomarker of interest or the cells by which it is expressed.

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Fig 1. Conformation and binding capability of GK1.5 cDb

(a) Schematic depicting an intact antibody (~150 kDa) and the cys-diabody (52 kDa), including the solvent-exposed disulfide bridge which is reduced for site-specific conjugation. (b) Size exclusion chromatography of GK1.5 cDb (dashed line), soluble murine CD4 (solid line), and soluble murine CD4 in complex with GK1.5 cDb following incubation at a 2:1 molar ratio (dotted line), with elution times in minutes noted above peaks. Arrows indicate elution of markers albumin (66 kDa) at 20.0 minutes and carbonic anhydrase (29 kDa) at 24.0 minutes. (c) Affinities of intact GK1.5 Ab and GK1.5 cDb were estimated with

ELISA, using immobilized soluble CD4 antigen (n=3 independent experiments; results of one representative experiment shown).

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Fig 2. Effect of GK1.5 cDb protein dose on anti-CD4 immunoPET imaging

(a) Representative coronal and (b) transverse microPET images from ^{89}Zr -malDFO-GK1.5 cDb dose escalation study. C57BL/6 mice were injected with 2, 6, 12, or 40 μg ^{89}Zr -malDFO-GK1.5 cDb. Images acquired 20 hr post-injection are displayed as 25 mm (coronal) or 2 mm (transverse) maximum-intensity projections. One representative mouse from each group is shown. The 12 μg image is from a previously published study [19] and is reproduced here. Major visible lymph nodes and other organs are labeled: cervical LN (CLN), axillary LN (ALN), spleen (Sp), liver (Li), kidney (K), inguinal LN (ILN), bone (B), popliteal LN (PLN).

Fig 3. Ex vivo biodistribution and target:muscle ratios from ^{89}Zr -malDFO-GK1.5 cDb dose escalation immunoPET study

(a) Following the PET scan 20 hr post-injection of probe, organs were removed, weighed, and counted to determine activity in each organ (expressed as % ID/g). Complete biodistribution data are provided in Table 1. Data for the 12 µg group are from a previously published study [19]; CT scanning was unavailable for the 12 µg group. (b) Tissue:muscle ratios for secondary lymphoid organs including spleen and axillary, inguinal, and cervical lymph nodes from biodistribution data (n=4 for 2, 6, and 40 µg protein doses; n=3 for 12 µg protein dose).

Fig 4. *In vivo* changes in cell populations and surface expression of CD4 by T cells in lymphoid organs post-administration of high-dose GK1.5 cDb

Flow cytometric analysis of T cells from mice, one or three days following intravenous administration of 40 µg GK1.5 cDb. CD4 was assayed with both PE-anti-CD4 antibody (clone RM4-4; panels a-b) and BV421-anti-CD4 antibody (clone GK1.5; panels d-e). (a, d) Change in CD4 expression by CD45⁺CD4⁺ T cells as measured by mean fluorescence intensity in mouse blood, spleen, ILN, and thymus, one or three days after GK1.5 cDb administration. (b, e) Percent of CD45⁺CD4⁺ T cells in mouse blood, spleen, ILN, and thymus, one or three days after GK1.5 cDb administration. (c) Percent of CD45⁺CD3⁺ T cells in mouse blood, spleen, ILN, and thymus, one or three days after GK1.5 cDb administration. Experimental groups were compared individually to the control untreated group (n=3–4 mice/group). * p<0.05, ** p<0.005, *** p<0.005.

Fig 5. *In vitro* effects of GK1.5 cDb on T cell proliferation and function

CFSE-labeled OT-II CD4⁺ T cells were cultured with OVA-pulsed splenocytes and incubated with various concentrations of GK1.5 cDb. (a) Proliferation was analyzed with flow cytometry 72 hours after addition of GK1.5 cDb, and percent divided was calculated by determining the percent of CFSE-labeled cells that divided at least once. (b) Replication index was determined by the fold-expansion of proliferative OT-II cells ($n = 3$ independent experiments). (c) Culture supernatant was collected at 72 hours and concentration of IFN γ was assayed ($n = 2$ independent experiments). All concentrations of GK1.5 cDb were compared to control well. n.s.: not significant, * $p < 0.05$, ** $p < 0.005$.

Fig 6. *In vivo* effects of GK1.5 cDb on T cells

(a) Change in surface CD4 expression by T cells from mice, one day following intravenous administration of 2 or 40 µg GK1.5 cDb (n=4 mice/group). Experimental groups were compared individually to control untreated group. (b) Percent OT-II cells divided in ILN and spleens of OVA-stimulated mice was assessed 72 hours after administration of GK1.5 cDb (n=3 mice/group). * p<0.05, ** p<0.005, *** p<0.0005, **** p<0.0001. Experimental groups were compared individually to control (OVA + T cells only) group. (c) Representative CFSE histograms of OVA-stimulated OT-II CD4⁺ T cells from spleen and ILN 72 h after administration of GK1.5 cDb. Dashed line indicates undivided cells.

Table 1*Ex vivo* biodistribution of ^{89}Zr -malDFO-GK1.5 cDb dose escalation immunoPET study

Organ	Percent injected dose per gram (%ID/g)			
	2 μg GK1.5 cDb	6 μg GK1.5 cDb	12 μg GK1.5 cDb	40 μg GK1.5 cDb
Blood	0.21 $\pm$ 0.03 *	0.29 $\pm$ 0.05	0.33 $\pm$ 0.01	0.36 $\pm$ 0.01 *
ALN	53 $\pm$ 9.3	47 $\pm$ 8.5	45 $\pm$ 1.4	39 $\pm$ 9.0
ILN	106 $\pm$ 13 *	70 $\pm$ 13	45 $\pm$ 5.3	48 $\pm$ 9.9
CLN	49 $\pm$ 7.6	72 $\pm$ 18	56 $\pm$ 6.4	50 $\pm$ 18
Spleen	70 $\pm$ 1.4 *	46 $\pm$ 5.0 *	30 $\pm$ 3.4	28 $\pm$ 1.1
Stomach	0.75 $\pm$ 0.31	1.44 $\pm$ 0.86	1.6 $\pm$ 0.49	1.1 $\pm$ 0.30
Intestines	2.4 $\pm$ 0.07	3.0 $\pm$ 0.30	3.1 $\pm$ 0.59	3.4 $\pm$ 0.31
Liver	4.4 $\pm$ 0.26 *	7.2 $\pm$ 0.47 *	11 $\pm$ 0.68	12 $\pm$ 0.6
Kidney	39 $\pm$ 6.7 **	69 $\pm$ 9.2 *	117 $\pm$ 8.7	129 $\pm$ 6.8
Thymus	1.1 $\pm$ 0.08	1.9 $\pm$ 0.21	2.7 $\pm$ 0.73	4.4 $\pm$ 0.83
Heart	0.56 $\pm$ 0.03 **	0.99 $\pm$ 0.17 *	1.8 $\pm$ 0.05	1.5 $\pm$ 0.12 *
Lung	1.02 $\pm$ 0.08 **	1.6 $\pm$ 0.22 *	2.4 $\pm$ 0.13	2.4 $\pm$ 0.28
Muscle	0.19 $\pm$ 0.02	0.36 $\pm$ 0.03	0.72 $\pm$ 0.15	0.58 $\pm$ 0.17
Bone	4.2 $\pm$ 1.1	4.1 $\pm$ 0.31	3.6 $\pm$ 0.37	4.1 $\pm$ 0.12
Carcass	0.59 $\pm$ 0.05 **	0.81 $\pm$ 0.08 **	1.3 $\pm$ 0.06	1.1 $\pm$ 0.09 *
Tail	5.2 $\pm$ 2.4	5.0 $\pm$ 1.1	7.9 $\pm$ 1.4	6.7 $\pm$ 0.76
Ratios				
Spleen:Muscle	341 $\pm$ 62	166 $\pm$ 48	91 $\pm$ 11	78 $\pm$ 3.8
ALN:Muscle	257 $\pm$ 65	168 $\pm$ 28	137 $\pm$ 6.3	110 $\pm$ 25
ILN:Muscle	539 $\pm$ 27	254 $\pm$ 64	138 $\pm$ 18	134 $\pm$ 31
CLN:Muscle	243 $\pm$ 71	252 $\pm$ 20	171 $\pm$ 23	141 $\pm$ 54

The 2, 6, and 40 μg groups (n=4 mice/group) were compared individually to the 12 μg group (n=3), data from which has been previously published [19]. Values are represented as mean $\pm$ standard deviation.

*
p<0.05

**
p<0.005

p<0.0005

Abbreviations: axillary LN (ALN), inguinal LN (ILN), cervical LN (CLN).