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# Development of an anti-guinea pig CD4 monoclonal antibody for depletion of CD4+ T cells *in vivo*

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A B S T R A C T

The guinea pig serves as a useful animal model for a number of human diseases and has played an important role during development and testing of experimental vaccines and disease therapies. However, the availability of reagents to examine the immunological response in this species is very limited. Monoclonal antibodies (mAb) specific for cell surface proteins or products of immune cells have been useful tools for characterizing and quantifying immune responses in humans and in murine models of human disease, but very few similar reagents are available for characterizing and manipulating the immune response of guinea pigs. A rat IgG2a mAb specific for guinea pig CD4 has previously been described and was shown to inhibit T cell proliferation, but was inefficient at depleting CD4+ T cells *in vivo*. We hypothesized that the *in vivo* CD4+ T cell depletion function of this mAb could be improved by expression of the rat IgG2b heavy chain. We show that the purified mAb from an IgG2b class-switch variant, but not the parental IgG2a mAb, significantly depleted CD4+ T cells from secondary lymphoid tissue of guinea pigs. Further, treatment of guinea pigs with the IgG2b mAb at 2.0 mg/kg resulted in depletion of CD4+ T cells from peripheral blood and spleen. The use of this modified antibody to specifically alter the immune response of guinea pigs should prove useful in a number of guinea pig infectious disease models.

## 1. Introduction

The guinea pig serves as an important model of human infectious diseases and vaccine testing. Historically, Robert Koch utilized guinea pigs to identify the causative agent of tuberculosis and more recently, the immune response to *Mycobacterium tuberculosis* and the efficacy of candidate vaccines have been examined extensively in this species (McMurray, 2001; Orme, 2005; Smith et al., 1970). Guinea pigs also serve as an important model for other human pathogens such as *Chlamydia* (Barron et al., 1979; Lamont et al., 1978), *Treponema pallidum* (Pierce et al., 1983; Wicher and Wicher, 1989), herpes simplex virus (Stanberry, 1991; Valencia et al., 2013), cytomegalovirus (Schleiss and McVoy, 2010), and emerging viral pathogens such as Lassa virus (Bell et al., 2017) and Ebola virus (Wong et al., 2015). Many guinea pig immune-related genes, including genes for IL8, IL12 (p35 and p40), RANTES, CD1 proteins, CD8, guinea pig leukocyte antigens, and many complement component genes (Antczak, 1982; Brade et al., 1972; Campbell et al., 1997; Hamuro et al., 1978; Hiromatsu et al., 2002; Nagarajan et al., 2004; Nicholson and Austen, 1977; Seya et al., 1991; Shiratori et al., 2001; Yoshimura and Johnson, 1993), are similar to their human counterpart genes. However, the immune response of guinea pigs to many pathogens has been incompletely examined because, compared to the vast array of reagents available to examine immunity in mice, only limited reagents exist for the study of immune responses in guinea pigs. This has limited the usefulness of these guinea pig models of infectious diseases.

In the absence of guinea pig strains deleted of specific immune genes, *in vivo* depletion of specific lymphocyte subsets by injection of specific antibody would be useful to determine the role of these cells in pathogenesis or protection in infectious disease models. Historically, in murine models, this approach resulted in specific depletion of targeted cells *in vivo* (Ghobrial et al., 1989; Le Gros et al., 1983) and was used to define the function of specific immune cell subsets in cell-mediated and humoral immune responses (Coulie et al., 1985; Kelley et al., 1987; Wofsy et al., 1985). Antibody-mediated *in vivo* cell depletion has also been used to define both pathogenic and protective roles of specific cell populations in a number of murine infectious disease models (Matloubian et al., 1994; Milligan et al., 1998; Nakane et al., 1991; Titus et al., 1985). Unfortunately, no antibody has been available to specifically deplete guinea pig T lymphocytes *in vivo*.

A B cell hybridoma (H155) secreting rat IgG2a mAb specific for guinea pig CD4 has been produced from rats immunized with purified guinea pig T lymphocytes (Schafer and Burger, 1991). While this H155 mAb can be used to deplete CD4+ T cells *in vitro* and could impair

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antigen-, alloantigen- and mitogen-driven proliferative responses of T cells, administration to guinea pigs does not result in effective depletion of CD4+ T cells (Dr. Roger Rank, University of Arkansas School of Medicine, personal communication). Many effector functions of IgG antibodies are associated with the specific IgG subclass expressed by the antibody (Kipps et al., 1985; Steplewski et al., 1985). To determine if a different IgG subclass would prove more effective for cell-specific depletion *in vivo*, we selected subclass switch variants of the rat IgG2a-producing parental H155 hybridoma (H155-IgG2a) that expressed the IgG2b Fc region, and tested the subclass switch variants for the ability to deplete CD4+ T cells *in vitro* and *in vivo*. Administration of this new anti-guinea pig CD4 IgG2b mAb to guinea pigs resulted in nearly complete depletion of CD4+ T lymphocytes. Continued treatment of guinea pigs with the H155-IgG2b mAb resulted in prolonged depletion of CD4+ T cells. To our knowledge, this is the first antibody reagent capable of effectively depleting the majority of CD4+ T lymphocytes in guinea pigs and it should be important for determining the function of CD4+ T cells in a number of guinea pig infectious disease models.

## 2. Materials and methods

### 2.1. Animals

Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) were housed in an AAALAC-approved vivarium and allowed to acclimate prior to use. All animal studies were approved by the UTMB Institutional Animal Care and Use Committee with oversight of staff veterinarians.

### 2.2. Screening of hybridoma clones

The approach for detecting and cloning hybridoma class switch variants from the H155 rat anti-guinea pig CD4 (IgG2a) hybridoma was modified from the method of Spira et al., (Spira et al., 1984) and is shown in Table 1. Briefly, H155 (rat IgG2a) cells were cultured at 500 cells/well in 96-well tissue plates. Supernatants from confluent cultures were screened for the presence of rat IgG1 by ELISA using goat anti-rat IgG1 as the capture antibody (Southern Biotech, Birmingham, AL). Plates were incubated with HRP-conjugated goat anti-rat IgG1 (Southern Biotech), developed, and the $OD_{450}$ was determined using a VersaMAX plate reader and SoftMAXPro 7.0.3 software as described previously (Milligan and Bernstein, 1995). Cells from individual wells showing the highest $OD_{450}$ reading were re-plated at 100 cells/well on additional 96 well plates and IgG1+ wells were identified by ELISA. The supernatant screening and cell subculture process was repeated at 10 cells/well followed by cloning of IgG1-producing cells by limiting dilution. IgG2b-secreting, spontaneous switch variants were detected from the H155-IgG1 cultures using a rat IgG2b capture ELISA for screening and were cloned by limiting dilution.

**Table 1**
Work-flow for detection and cloning of Fc switch variants of the H155 anti-guinea pig CD4 hybridoma.

- Subculture H155-IgG2a hybridoma (1000 cells/well).
- Test subculture supernatants for IgG1 antibody by IgG1 capture ELISA.
- Pick $IgG1^+$ wells and increase frequency of IgG1 subclass switch variants by culture at decreasing concentrations of hybridoma cells (100, then 10 cells/well).
  - $IgG1^+$ wells identified by IgG1 capture ELISA.
- Clone H155-IgG1 subclass switch variant hybridoma by limiting dilution.
- Culture H155– IgG1-secreting hybridoma (1000 cells/well).
- Test culture supernatants for IgG2b mAb by IgG2b capture ELISA.
- Pick $IgG2b^+$ wells. Increase frequency of IgG2b-producing subclass switch variants by culture at decreasing cell concentrations (100, then 10 cells/well).
- Identify $IgG2b^+$ wells by capture ELISA.
- Clone H155-IgG2b mAb by limiting dilution.

### 2.3. Antibody production

Hybridoma clones were cultured in serum-free hybridoma culture medium (DMEM plus 1% L-glutamine, 1% non-essential amino acids, 1% penicillin/streptomycin and 1% Nutridoma-SP [Roche Diagnostics, Mannheim, Germany]). The antibody-containing supernatant was clarified by centrifugation and antibodies were precipitated with ammonium sulfate. Precipitated protein was pelleted by centrifugation, renatured by addition of PBS and dialyzed extensively against PBS. The dialyzed antibody was concentrated using Centriprep filter units (Merck Millipore Ltd., Tullagreen, Ireland) and IgG antibody was purified using a NAb™ Protein G Spin Kit (ThermoScientific, Rockford, IL) and desalted using buffer exchange Zeba™ Spin Desalting Columns (ThermoScientific) following the manufacturer's instructions. IgG antibody was again concentrated with Centriprep filter units, sterilized by 0.02 μm filtration, and stored at 4 °C.

### 2.4. Antibody quantification

Two methods were employed to quantify purified IgG preparations. Purified protein in mAb preparations was quantified using the Bio-Rad DC™ Protein Assay kit II (Bio-Rad Laboratories, Inc., Hercules, CA) following the manufacturer's instructions. Serial dilutions of protein standards and purified IgG solutions were read at 560 nm, and compared to a purified protein standard curve using SoftMAX Pro software. A quantitative IgG ELISA was also used to verify the protein concentration of the targeted antibody. ELISA plates were coated with goat anti-rat IgG2b (Southern Biotech). Purified rat IgG2b standards and purified IgG2b preparations were plated and incubated overnight at 4 °C. Plate-bound IgG2b was detected by the addition of goat anti-rat IgG2b HRP (Southern Biotech). Plates were developed and read at 450 nm. The IgG2b concentration of purified preparations was determined by comparing the $OD_{450}$ against that of the standard curve using SoftMAX Pro software.

### 2.5. In vitro depletion

Guinea pig spleen cells were suspended in 1.0 ml of Hank's Balanced Salt Solution (HBSS) with 5% newborn calf serum (NBCS) in the presence of various concentrations of H155-IgG2a mAb, H155-IgG2b mAb, or medium only as a control. After a 30 min incubation at 4 °C, cells were pelleted and suspended in 1.0 ml of HBSS + NBCS with a 1:6 or 1:12 dilution of Low Tox Rabbit complement (Cedarlane Laboratories, Burlington, Ontario, Canada) or medium only as a control and incubated at 37 °C for 30 min. To ensure that mAb-treated CD4+ cells were actually depleted rather than undetectable due to masking of surface CD4 by bound anti-CD4 mAbs, the antibody-treated splenocytes were washed free of antibody and cultured for 24 h to allow regeneration of surface CD4 prior to flow cytometric analysis. Cells were stained for CD4+ and CD8+ T cells as described previously (Bourne et al., 2019). Data were acquired on a BD FACSCanto II (BD Biosciences, San Jose, CA) at the UTMB Flow cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

### 2.6. In vivo depletion

Guinea pigs were injected intraperitoneally with 5 mg/kg of the parental rat IgG2a H155 mAb, the subclass variant H155-IgG2b mAb, or dilute rat serum as a control. In initial experiments, animals were treated at 5.0 mg/kg on day 0 and day 3 before testing depletion of $CD4^+$ T cells by flow cytometry on day 8. To investigate the effectiveness of the antibody at a lower dose, animals were treated with 2.0 mg/kg anti-CD4 IgG2b or rat serum as control on days 0, 3, and 7. In all experiments, isolated cells were cultured overnight prior to surface staining for CD4 and CD8, to allow regeneration of any surface markers that might have been potentially masked by *in vivo* antibody treatment.

Flow cytometric analysis of cells from spleen, mesenteric lymph nodes (mLN) inguinal LN (ingLN), and peripheral blood (PBL) to quantify CD4+ cell depletion was performed as described previously (Bourne et al., 2019).

### 2.7. Statistical analysis

Statistical differences for antibody treatments were determined using an unpaired, two-tailed Student *t*-test with Welch's correction. Values for $P < .05$ were considered significant. Statistical calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Depletion of guinea pig CD4+ T cells in vitro

We hypothesized that changing the IgG subclass expressed by the parental H155-IgG2a mAb to IgG2b would improve the cytotoxic properties of the mAb. To compare the ability of the H155-IgG2b switch variant mAb to deplete CD4+ cells *in vitro* with the parental H155-IgG2a mAb, guinea pig splenocytes were incubated with the individual mAbs, or medium only as a control, then washed and incubated in the presence of complement. Depletion of CD4+ T cells in mAb-treated cells was calculated relative to untreated (no antibody) cells. As shown in Table 2, although the depletion was not complete, both the parental H155-IgG2a and the H155-IgG2b subclass switch variant mAb depleted > 75% of the CD4+ T cells from guinea pig splenocyte populations in the presence of the highest concentration of complement (1:6 dilution). Lower levels of depletion (~42–58%) were achieved by treatment with either mAb in the presence of a lower concentration of complement (1:12 dilution). Together, these results demonstrate that both the parental H155-IgG2a and the H155-IgG2b subclass switch variant mAb possessed equivalent cytolytic activity against guinea pig CD4+ T cells *in vitro* in the presence of complement.

### 3.2. Depletion of guinea pig CD4+ T cells in vivo by intraperitoneal injection of the H155-IgG2b IgG subclass switch variant

*In vivo* cell depletion by injection of mAb may involve different mechanisms than *in vitro* depletion and has been shown to be influenced by the expression of specific IgG subclasses (Kipps et al., 1985; Steplewski et al., 1985). We hypothesized that the H155 IgG2b should deplete more efficiently than the parental H155-IgG2a as a reflection of the biological functions of the two Fc regions. To test the depletion activity *in vivo*, guinea pigs were injected intraperitoneally with 5.0 mg/kg purified H155-IgG2a or the switch variant H155-IgG2b mAb on days 0 and 3. CD4+ cells in the spleens, mesenteric lymph nodes (LN) and inguinal LN were detected by flow cytometry on day 8 and the percent depletion relative to cells from control-treated animals was calculated. Fig. 1 shows the gating strategy for the flow cytometric analysis of splenocytes from antibody-treated animals. The CD4+ T cells detected in spleens from a control-treated animal, an H155-IgG2b mAb-treated animal, and an H155-IgG2a mAb-treated animal are marked by an oval in the CD4 *vs* CD8 histograms. The results show nearly complete depletion of CD4+ T cells in the H155-IgG2b mAb-treated animal compared to the control-treated animal. Additionally, CD4 T cell depletion in the parental H155-IgG2a mAb-treated animals was markedly less efficient compared to depletion in the subclass switch variant H155-IgG2b mAb-treated animals. Table 3 shows that treatment with H155-IgG2b mAb resulted in depletion of 98.9 ± 0.3, 96.1 ± 2.3, and 83.5 ± 17.7% of CD4+ cells from the spleen, mesenteric LN, and inguinal LN, respectively, compared to control-treated animals. Treatment with the parental H155-IgG2a mAb resulted in depletion of CD4+ T cells from these tissues that ranged between ~8–20%. These results demonstrate the effective *in vivo* depletion of CD4+ cells from guinea pigs by treatment with the newly developed H155-IgG2b mAb relative to depletion by treatment with the parental H155 anti-guinea pig CD4 IgG2a mAb. CD4+ cell depletion from spleen, mLN, and ingLN by the H155 IgG2b mAb could be maintained at approximately 95% depletion for up to 21 days compared to control-treated animals demonstrating the utility of this mAb for longer-term depletion studies [G. Milligan unpublished data and (Bourne et al., 2019)].

To determine if delivery of a lower antibody dose was effective at *in vivo* depletion, we injected guinea pigs with H155 IgG2b mAb at 2.0 mg/kg. As shown in Table 4, animals receiving two or three treatments were depleted of approximately 96–99% of CD4+ cells from the spleen and peripheral blood on days 7 and 11 after treatment initiation. Together, these results demonstrate effective depletion of CD4+ cells from peripheral blood and secondary lymphoid tissues of guinea pigs that can be extended for at least 21 days following multiple injections of the anti-guinea pig CD4 IgG2b mAb.

**Table 2**
*In vitro* depletion of $CD4^+$ T lymphocytes from guinea pig splenocytes using the parental H155-IgG2a and H155-IgG2b subclass switch variant mAb.

| % CD4 depletion[a] | | | |
|---|---|---|---|
| mAb | Complement | | |
| | 1:6 | 1:12 | None |
| H155-IgG2b | | | |
| 5 μg/ml | 76.9 ± 10.1[b] | 58.8 ± 4.5 | 9.9 ± 14.0 |
| 0.5 μg/ml | 78.1 ± 7.3 | 57.4 ± 5.6 | 11.6 ± 16.4 |
| H155-IgG2a | | | |
| 5 μg/ml | 78.0 ± 3.9 | 44.8 ± 5.3 | 0 ± 0 |
| 0.5 μg/ml | 76.6 ± 7.6 | 42.7 ± 6.1 | 6.45 ± 1.8 |

[a] % CD4+ cell depletion calculated as [1 − (% $CD4^+$ cells in mAb + C treated culture/% $CD4^+$ cells in C treatment only cultures)] × 100.
[b] Results shown are the mean ± SD of two identical experiments.

## 4. Discussion

Many infectious diseases are best modeled in guinea pigs, but the lack of immune reagents to characterize the guinea pig immune response has limited the usefulness of the models. Prior to the development of mouse strains with targeted deletion of immune genes, treatment of mice with mAb specific for immune cell surface markers provided important information about the function of immune cells expressing the targeted proteins in protective immune responses. The availability of mAb specific for markers of T lymphocyte subsets of guinea pigs is limited and generally, these mAb have not been suitable for *in vivo* depletion approaches to probe T cell function in infectious disease models. The results of our current studies demonstrate the development of an anti-guinea pig CD4+ mAb for *in vivo* depletion of CD4+ T lymphocytes.

The majority of hybridoma cells stably secrete IgG antibodies of a single subclass; however, individual hybridoma cells can spontaneously switch to expression of a downstream Fc region, although at very low frequency (Preud'Homme et al., 1975). To test if altering the IgG subclass of a rat IgG2a-expressing mAb might enhance *in vivo* depletion of guinea pig CD4+ T cells *in vivo*, we selected for and isolated a spontaneous IgG subclass switch variant hybridoma that retained the original Fab region required for binding to guinea pig CD4 but that expressed the IgG2b subclass. Treatment of splenocytes with either the H155-IgG2b variant or the parental H155-IgG2a mAb in the presence of complement resulted in partial depletion of CD4+ T cells *in vitro*. This depletion required the presence of complement and the level of depletion was comparable between the two mAb. However, the two mAb differed in their ability to deplete CD4+ T cells following injection of guinea pigs (Fig. 1, Table 3). Administration of the parental H155-

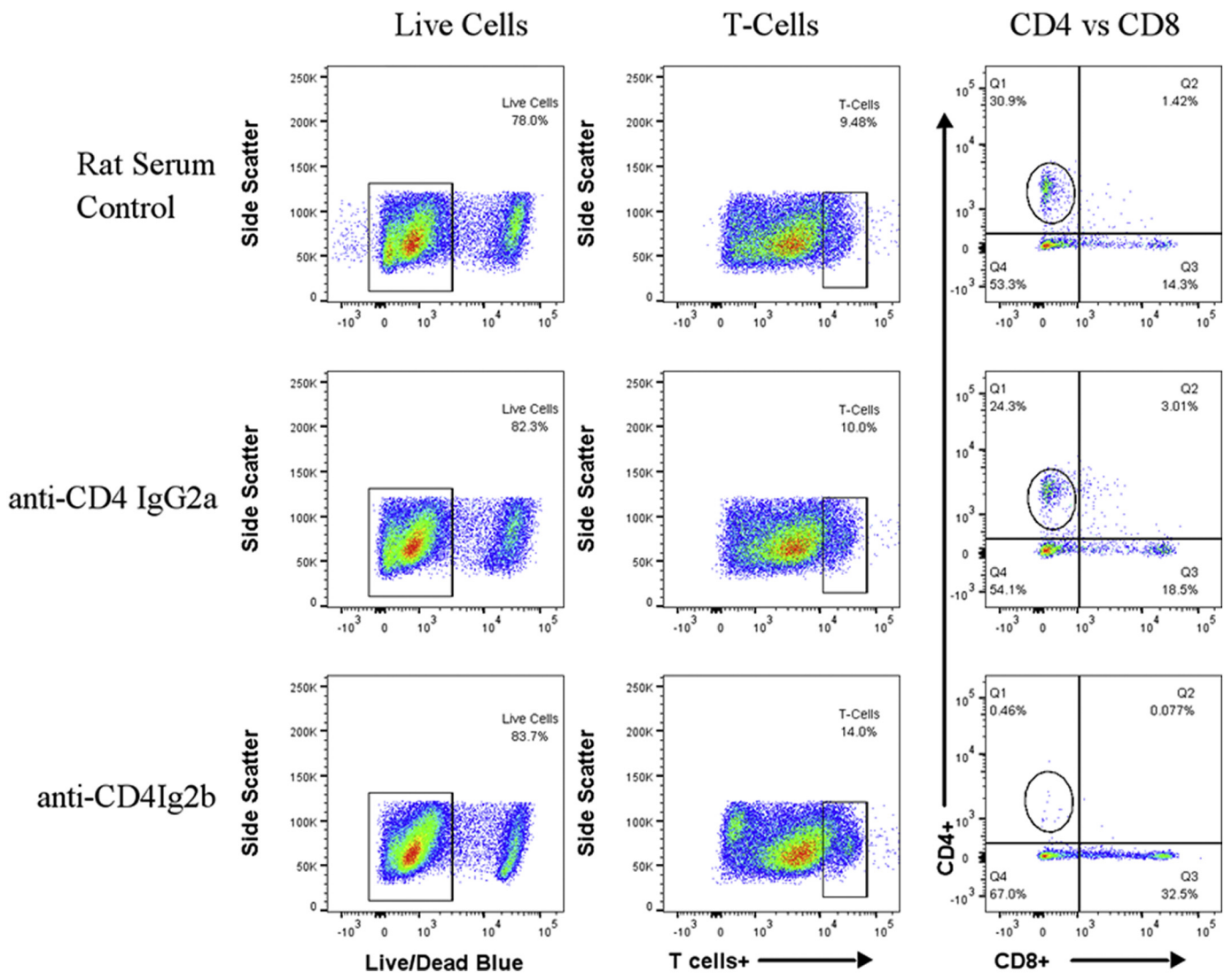


**Fig. 1.** Depletion of CD4+ T cells from secondary lymphoid tissues of guinea pigs by treatment with the anti-guinea pig CD4 mAb, H155-IgG2b. Representative histograms demonstrating the gating strategy for analysis of CD4+ and CD8+ T lymphocyte populations isolated from lymphoid tissues. Results are from a single, representative experiment of two performed.

**Table 3**
Comparison of *in vivo* $CD4^+$ cell depletion by treatment with anti-guinea pig CD4 H155-IgG2a and H155-IgG2b mAbs.

| Tissue | mAb Treatment[a] | % CD4 Depletion[b] |
|---|---|---|
| Spleen | H155-IgG2b | 98.9 ± 0.3 |
| | H155-IgG2a | 20.1 ± 4.3 |
| Mesenteric LN | H155-IgG2b | 96.1 ± 2.3 |
| | H155-IgG2a | 16.5 ± 1.0 |
| Inguinal LN | H155-IgG2b | 83.5 ± 17.7 |
| | H155-IgG2a | 8.1 ± 11.5 |

[a] Animals received 5 mg/kg of purified mAb or were control-treated on days 0 and 3. Depletion of $CD4^+$ cells was tested by flow cytometry on day 8. Results shown are from two guinea pigs receiving H155-IgG2b mAb and two guinea pigs receiving H155-IgG2a mAb compared to results from two control-treated animals.

[b] % depletion calculated as [1 − (%$CD4^+$ cells in tissue of H155 mAb treated animal ÷ % $CD4^+$ cells in tissue of control-treated animal)] × 100. Results are expressed as the mean ± SD values from two animals.

IgG2a mAb resulted in only partial depletion of CD4+ T cells from the spleen, inguinal LN, or mesenteric LN. However, the H155-IgG2b variant resulted in nearly complete depletion of CD4+ T cells from these tissues. In further support of the utility of this cell depletion approach, we recently showed sustained depletion of CD4+ T cells from secondary lymphoid tissues and peripheral tissues for up to 21 days by treatment with this novel antibody, resulting in an increase in the mean number of HSV-2 shedding days compared to control-treated animals

**Table 4**
Effect of decreasing effective antibody treatment dose on depletion of CD4+ cells.

| Tissue | Day of treatment[a] | % CD4 depletion[b] |
|---|---|---|
| PBL | 7 | 99.7 ± 0.1 |
| | 11 | 95.8 ± 2.9 |
| Spleen | 7 | 99.4 ± 0.8 |
| | 11 | 93.7 ± 5.3 |

[a] Guinea pigs were treated with 2.0 mg/kg H155 IgG2b or control-treated on days 0, 3, 7. Flow cytometric analysis to quantify CD4+ cell depletion was performed from cells harvested on days 7 and 11 of mAb treatment.

[b] % CD4+ cell depletion calculated as: [1 − (% CD4+ cells in tissue from mAb-treated animal ÷ % CD4+ cells in tissue from control- treated animal)] × 100. Results shown are the mean ± SD from two animals per time point.

(Bourne et al., 2019).

IgG antibodies are generally superior to IgM antibodies for *in vivo* depletion techniques (Kruisbeek, 2001). Additionally, the expression of specific IgG subclasses has been shown to be very important in the therapeutic activity of mAb (Kipps et al., 1985; Steplewski et al., 1985) and *in vivo* depletion of specific T cell subsets (Waldor et al., 1987). Biological activities such as complement fixation or the ability to interact with specific Fcγ receptors involved in antibody-dependent cell mediated cytotoxicity (ADCC) are dependent upon the IgG subclass of the monoclonal antibody. Rat antibodies expressing the IgG2b subclass fix complement and have been shown to mediate greater levels of complement-mediated lysis in the presence of guinea pig complement compared to rat IgG2a antibody (Bruggemann et al., 1989; Pluschke et al., 1989). Additionally, rat IgG2b has been shown to mediate greater ADCC activity than antibodies expressing the IgG2a subclass (Bruggemann et al., 1989). Rat IgG2a and IgG2b antibodies have also been shown to bind to distinct FcγR on macrophages which may affect functional activity including ADCC and opsonization (Boltz-Nitulescu et al., 1981; Miklos et al., 1993). Differences in effector function between the two IgG subclasses are likely critical in the ability to mediate antibody-mediated depletion of specific immune cells although the exact mechanisms underlying this phenomenon *in vivo* are uncertain,

Guinea pigs are utilized as accurate models of several infectious diseases due to the similarities in disease pathogenesis between humans and guinea pigs. Additionally, given the similarities in placental structure between humans and guinea pigs, these animals serve as an appropriate model for intrauterine infections (Schleiss and McVoy, 2010). The depletion qualities of this newly developed anti-guinea pig CD4 mAb strongly suggest that H155-IgG2b mAb will be extremely useful in the infectious disease field. *In vivo* depletion experiments utilizing this mAb should help elucidate the role of CD4+ T cells in protection and pathogenesis in a number of infectious disease models in this species.

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