CD8$^+$ T-CELL RESPONSE POTENTIAL, AS DETERMINED BY

EXPRESSION OF THE HIGH AFFINITY INTERLEUKIN-2 RECEPTOR, IN

MURINE AIDS

By

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ABSTRACT

Human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS), which can follow infection with the virus, is a major health issue worldwide. Murine AIDS (MAIDS) is a model system used for the study of AIDS. By comparing the immune response to murine leukemia virus of MAIDS resistant and susceptible strains of mice, and making observations of the features of MAIDS, we can hopefully gain insight into ways of approaching treatment and prevention of HIV and AIDS in humans.

Studies have shown that CD8$^+$ T-cells are important for resistance to MAIDS and human AIDS, and it has been proposed that it is the level of CD8$^+$ T-cell activation which is important (Makino *et al.*, 1992; Tang *et al.*, 1997). Interleukin-2 (IL-2) is one of the key cytokines involved in the activation of T-cells. Since CD8$^+$ T-cells require a stronger IL-2 signal than CD4$^+$ T-cells to be activated, they are more likely to be affected by decreases in levels of IL-2. T-cells can express both high and low affinity IL-2 receptors. The low affinity receptor expressed on naïve T-cells is comprised of two subunits, beta and gamma, while the high affinity receptor on activated T-cells is made up of the beta and gamma subunits, along with an alpha subunit (CD25).

This study used flow cytometry to compare the number of CD8$^+$ T-cells expressing high affinity IL-2 receptors in MAIDS susceptible and resistant mice. CD8$^+$ T-cells were isolated from the spleen and stained with a fluorescently-labeled antibody specific to the high affinity-specific alpha subunit of the IL-2 receptor (CD25). Results indicate that BALB/c and C57BL/6 mice may have different expression patterns of CD25 on CD8$^+$ T-cells following infection. In addition, the percent of spleen leukocytes expressing CD25 was found to be higher in 2-week infected C57BL/6 mice than in 2-week infected BALB/c mice.

INTRODUCTION

<u>HIV and AIDS</u>

Human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS), which follows infection with the virus, is a major health issue worldwide, and at present there is no known cure or vaccine against the disease. In 2005, nearly five million new HIV infections occurred, and approximately three million people died from AIDS; today there are over 40 million people living with HIV (unaids.org, 2005).

HIV belongs to a class of retroviruses known as lentiviruses, which are characterized by the slow progression of the diseases they cause. Retroviruses possess a RNA genome which, upon infection of a host cell, is copied into complementary DNA (cDNA) and then integrated into the host DNA. The RNA transcripts that are subsequently produced are translated into viral proteins, as well as create RNA genomes for new viral particles (Janeway *et al.*, 2005). HIV contains two copies of the RNA genome, each of which consists of nine genes including *gag, pol, and env* (Parham, 2005). The *gag* gene encodes for the structural proteins of the virus, *pol* encodes for the enzymes needed for viral replication and integration, and *env* encodes the glycoproteins of the viral envelope (Janeway *et al.*, 2005). Other genes such as *tat* and *rev* serve to promote the replication of the virus in activated T-cells (Janeway *et al.*, 2005). HIV uses the CD4 molecule as a receptor on human cells, making CD4 T-cells, as well as

macrophages and dendritic cells, susceptible to infection and CD4 T-cells become the major source of virus production throughout infection (Janeway *et al.*, 2005).

When an individual is first infected with HIV, the amount of virus in the peripheral blood becomes abundant and the number of CD4 T-cells declines. This is followed by an HIV-specific immune response that leads to a reduction of the viral load, an increase in CD4 T-cell numbers, and movement into an asymptomatic period (Janeway *et al.*, 2005). However, despite the asymptomatic status of infected individuals, viral replication and infection persist, and in most cases infection with HIV will eventually progress to AIDS. A person is considered to have AIDS when the level of CD4 T-cells has dropped too low to mount a successful immune response, making the individual susceptible to opportunistic infections (Janeway *et al.,* 2005). Compromise of the immune system and cumulative damage from the HIV and other infections eventually leads to death.

Despite what we already know, there remains much about HIV and AIDS which is poorly understood. The development of successful drug therapies for the disease or a vaccine against the virus is a desirable, yet difficult, goal. Among other problems remains our uncertainty regarding what aspects of the immune response are most important for protective immunity against HIV (Janeway *et al.*, 2005). Thus, in order to work towards the goal of treating, curing, and preventing infection with HIV and its progression to AIDS, we need to continue increasing our knowledge about the disease.

MAIDS model system

As with any human disease, there are issues, ethical and otherwise, that affect the extent to which we can study HIV and AIDS in humans. While humans are the only species known to be susceptible to HIV, model systems have been developed in other animals by using retroviruses that produce characteristics and symptoms which parallel AIDS (Jolicoeur, 1991). One of these model systems is murine AIDS (MAIDS), induced by infection with a mixture of murine leukemia viruses (MuLV). The LP-BM5 MuLV mixture was first isolated by Duplan and Latarget, and contains replication-competent B-tropic ecotropic MuLV, mink cell focus-inducing MuLV, and a replication defective virus (Latarget and Duplan, 1962). Like HIV, MuLV is a retrovirus which infects a specific subset of immune cells, although in this case it is B-cells which are the primary source of virus expression (Green, 2001). The symptoms of MAIDS include lymphadenopathy and splenomegaly, impaired B-cell and T-cell response, and increased susceptibility to other infections; all of which are also characteristic of HIV infection in humans (Deslauriers *et al.*, 1997; Morse *et al.*, 1992).

Inbred mouse strains vary in the level to which they are susceptible to disease following infection with MuLV, ranging from complete disease resistance to high susceptibility (Morse *et al.*, 1992). BALB/c mice show resistance to disease following virus exposure and never develop any symptoms of MAIDS, whereas C57BL/6 mice are highly susceptible to disease, leading to the

development of MAIDS and consequent death (Jolicoeur, 1991; Morse *et al.*, 1992).

Upon infection, BALB/c mice mount a healthy immune response against MuLV and around two weeks after being infected their immune system begins to eradicate the virus, leading to a full recovery. In addition, after the first exposure to the virus they have immunological memory of the infection and are resistant to any future challenges with MuLV.

C57BL/6 mice, on the other hand, are unable to produce a directed immune response against MuLV and are unsuccessful at eliminating the virus. Changes to the C57BL/6 immune function are able to be seen as early as one week after infection and function is markedly reduced by 4 weeks after infection (Morse *et al.,* 1992). Responses to exogenous infections are noticeably altered by 7 weeks and mortality typically occurs by 24 weeks (Morse *et al.,* 1992). At one week after infection, B-cell activation and proliferation is observed along with CD4 T-cell proliferation and cytokine expression. As the disease progresses, proliferation continues, along with phenotypic changes to the cells corresponding to functional deficiency (Morse *et al.,* 1992).

By comparing the immune response to MuLV of MAIDS resistant and susceptible strains of mice, and making observations of the features of MAIDS, we can hopefully gain insight into ways of approaching treatment and prevention of HIV and AIDS in humans. This includes increasing our understanding of the

mechanisms by which immune function is lost as well as the crucial aspects of the immune response that allow for successful defense against viral infection.

Role of CD8^{+} T-cells

T-cells are one of the main classes of immune cells and are divided into two different subclasses, CD4^{+} T-cells and CD8^{+} T-cells, which are responsible for performing different functions in the immune system. The main function of CD4^{+} T-cells is to help other cells of the immune system respond to infections. For instance, they secrete cytokines to help direct the immune response and can help activate naïve CD8^{+} T-cells. The CD8^{+} T-cells are known as cytotoxic T-cells and are responsible for recognizing cells infected with viruses and killing the infected cells before the virus can replicate. They function by inducing apoptosis, programmed cell death, when they interact with infected cells. Apoptosis serves the purpose of both killing the infected cell, as well as activating nucleases which can degrade the viral DNA (Janeway *et al.*, 2005). In addition, CD8^{+} T-cells release cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which contribute to the immune defense.

CD8^{+} T-cell and AIDS

Studies have shown that CD8^{+} T-cells are important for resistance against MAIDS and human AIDS (Makino *et al.*, 1992; Tomaras *et al.*, 2000). In humans, it has been observed that there is an inverse relationship between the plasma viral load and the number of CD8^{+} T-cells specific for an HLA-A2-

restricted HIV peptide (Janeway *et al.*, 2005). It has also been shown that many asymptomatic individuals with HIV show strong CD8$^+$ T-cell mediated antiviral activities and that disease progresses slower for patients with higher levels of HIV-specific CD8$^+$ T-cells (Janeway *et al.*, 2005). There was also a small group of African women who were estimated to have been exposed to HIV many times a month for up to five years but did not develop the disease. These women were shown to lack antibody responses to HIV, but they had cytotoxic T-cell responses to HIV peptide epitopes (Janeway *et al.*, 2005).

Yet, even in the cases of CD8$^+$ T-cell response to HIV, individuals are unable to eradicate the virus and, except in the case of long-term nonprogressors, immunodeficiency eventually occurs. Studies show that freshly isolated CD8$^+$ T-cells specific to HIV are often unable to lyse HIV-infected cells (Lieberman *et al.*, 2001). Other properties besides cytotoxicity that have been proposed to play a role in the ineffective function of CD8$^+$ T-cells during HIV infection include impaired trafficking to the major sites of viral replication, no production of IL-2 or inability to respond to low IL-2 concentrations, and down-regulation of key molecules involved in T-cell signaling (Lieberman *et al.*, 2001)

CD8$^+$ T-cells and MAIDS

Studies in the MAIDS model have further confirmed the importance of CD8$^+$ T-cells in the study of immunodeficiency. The normally resistant A/J mice become susceptible to MuLV infection and the development of MAIDS when depleted of the CD8$^+$ subset of cells by treatment with anti-CD8 antibody

(Makino *et al.*, 1992). Makino *et al.* (1992) also found that when comparing mice depleted of either CD8^{+} cells, CD4^{+} cells, IL-2, or IFN-γ, the number of spleen cells producing ecotropic virus were highest in mice depleted of CD8^{+} cells (Makino *et al.*, 1992). In another study, resistant B10.A mice became susceptible when they lacked CD8^{+} T-cells due to knockout of the β2-microglobulin gene, which is essential for MHC class I expression (Tang *et al.*, 1997).

Additionally, it has been shown that the percentage of CD8^{+} T-cells is selectively decreased during MAIDS. Significant decreases in the frequencies of CD8^{+} T-cells were found in the spleen and lymph nodes of mice with MAIDS at 4 to 12 weeks post-infection, and in the peripheral blood at approximately 6 to 10 weeks post-infection (Chau *et al.*, 1993; Morse *et al.*, 1989; Pavlovitch *et al.*, 1996; Yetter *et al.*, 1988). At 4 to 5 weeks post-infection, Morse *et al.* (1989) found the percentage of CD8^{+} T-cells in the spleens of MAIDS susceptible mice to be 6 percent; approximately 50 percent lower than the percent of CD8^{+} T-cells found in uninfected mice.

Preliminary cDNA microarray analysis in the Stranford laboratory has also found that at 7 days post-infection the expression of the alpha and beta chains of the CD8 molecule in C57BL/6 mice is ¾ the level of expression seen in BALB/c mice (Tepsuporn, 2005). However, though this decrease in CD8^{+} cells occurs during MAIDS, the study done by Tang *et al.* (1997) with β2-microglobulin knockout mice suggests that the frequency of CD8^{+} cells does not determine resistance or susceptibility to MAIDS, because heterozygous mutants

($\beta 2M^{+/-}$) express the same frequency of $CD8^+$ cells as the resistant wild-type mice

($\beta 2M^{+/+}$) but still develop the disease. It is thus proposed that the difference may

lie at the level of $CD8^+$ T-cell activation (Tang *et al.*, 1997).

Interleukin-2

The key cytokine involved in activation and acquisition of effector

functions of T-cells is interleukin-2 (IL-2). It has been shown that in *in vitro*

studies, T-cells from IL-2 deficient mice have impaired proliferation and effector

functions (Nelson, 2004). $CD8^+$ T-cells require stronger stimulation than $CD4^+$

T-cells to become activated (Parham, 2005). This means that they require more

IL-2 for activation and are more sensitive than other lymphocyte subsets to

decreases in the presence of IL-2.

In patients with HIV, endogenous IL-2 production is greatly reduced, as

well as expression of the IL-2 receptor (Dix and Cousins, 2004; Lieberman,

2001). Synthesis of IL-2 by T-cells is regulated by signals from the T-cell

receptor and CD28 (Nelson, 2004). It is a subpopulation of $CD8^+$ T-cells, which

are $CD28^-$, that is responsible for mediating HIV-specific cytotoxicity in infected

individuals (Trimble *et al.*, 2000). These cells produce IFN-γ, but fail to produce

IL-2 and have a reduced capacity for proliferation (Trimble *et al.*, 2000). In HIV

infected individuals, a large proportion of $CD8^+$ T-cells also have reduced

expression of CD3ζ, a key component of T-cell receptor signaling (Trimble *et al.*,

2000). Culturing these cells with IL-2 for only 6 to 8 hours leads to an increase in

CD3ζ along with HIV-specific cytotoxicity, supporting a role for IL-2 in the ability of T-cells to fight HIV (Trimble *et al.*, 2000).

IL-2 immunotherapy has been tested in clinical trials to help improve the immune responses of HIV-1 infected patients, and has been suggested as a possible therapy to protect against AIDS-related human cytomegalovirus and other opportunistic infections during HIV infection (Dix and Cousins, 2004). Administration of exogenous IL-2 to patients with HIV resulted in a reduction of the markers of abnormal T-cell activation, increases in naïve and memory T-cells, and no persistent increase in viral load was observed (Dix and Cousins, 2004).

Previous studies have also found a decrease in the level of IL-2 production in mice susceptible to MAIDS. Studies of one week infected C57BL/6 mice show IL-2 levels significantly higher than uninfected mice. However, at two weeks after infection, IL-2 levels were undetectable, and remained undetectable at any of the following time points (Gazzinelli *et al.*, 1992). Similar to HIV related studies involving cytotoxicity of CD8^{+} T-cells, supplementing spleen cells from 7-9 week infected mice *in vitro* with recombinant IL-2 has been shown to produce CTL (cytotoxic T-lymphocyte) activities near the level of equally treated cells from uninfected mice (Morse *et al.*, 1989). This suggests that up to at least 9 weeks post-infection, the CD8^{+} cells of mice with MAIDS retain the potential to become activated cytotoxic T-cells (Morse *et al.*, 1989).

Interleukin-2 Receptor

Naïve and activated T-cells express receptors of different affinities for IL-2; naïve T-cells express the low affinity IL-2 receptor, while activated T-cells express the high affinity IL-2 receptor (Parham, 2005). The high affinity IL-2 receptor consists of an alpha (CD25), beta (CD122), and gamma (CD132) subunit, while a lower affinity IL-2 receptor is formed by just the beta and gamma subunits (Minami *et al.*, 1993). While the low affinity receptor is capable of signaling, it appears that the high affinity receptor is most physiologically important as mice lacking CD25 show the same phenotype as mice deficient in IL-2 (Nelson, 2005).

Expression of the IL-2Rα (CD25) increases the affinity of IL-2 binding approximately 100 fold, which allows for IL-2 to be effective at much lower concentrations (Kim and Leonard, 2002). In addition, the alpha subunit creates the receptor's specificity for IL-2, since the beta and gamma subunits are also components of the IL-15 receptor (Church, 2003). There is a tight genetic control on the expression of the alpha subunit, and mature T-cell and B-cells will only start to express it after cytokine stimulation (Church, 2003).

Purpose and Expectations

The purpose of this study was originally to compare the expression of the high affinity IL-2 receptor on CD8^{+} T-cells of MAIDS resistant BALB/c mice and susceptible C57BL/6 mice at various time points after infection with MuLV. The

hope was that this would indicate if after infection there exist differences in the activation potential of CD8$^+$ T-cells between the two strains of mice. Lower expression of IL-2Rα on CD8$^+$ T-cells of C57BL/6 mice could be a sign of decreased activation potential. Since CD8$^+$ T-cells play an important role in resistance to MAIDS, the absence of a strong CTL response when infected with MuLV could be one facet leading to the development of MAIDS in susceptible strains.

The first step was to investigate the percentages of CD8$^+$ T-cells at one week after infection. This was to confirm that the decrease in percentage of CD8$^+$ T-cells cited in the literature begins to occur at earlier time points than previously studied. It was also to show the behavior of CD8$^+$ T-cells for the BALB/c mice, since the literature focused on the susceptible strains only.

I then isolated CD8$^+$ T-cells from naïve, one week, two week, and four week infected BALB/c and C57BL/6 mice to compare the expression of the high affinity IL-2 receptor, as determined by expression of the alpha subunit (CD25), between BALB/c and C57BL/6 at each of the time points. The time points I looked at were 1 week, 2 weeks, and 4 weeks post-infection. I chose 1 week because it is the time at which high levels of IL-2 are seen in C57BL/6 mice and 2 weeks because it is when studies show the IL-2 levels have dropped to undetectable (Gazzinelli *et al.*, 1992). I also chose 4 weeks post infection because this is when previous studies indicated a significant decrease in the percentage of CD8$^+$ cells in mice with MAIDS (Morse *et al.*, 1989).

My initial studies with isolated CD8$^+$ T-cells indicated that 2 weeks was the time point most interesting to pursue, so I attempted to repeat the experiment with biological replicates of each mouse strain. However, I was unable to isolate CD8$^+$ T-cells for these experiments and instead looked at CD25 expression on all spleen leukocytes for 2 week infected mice.

MATERIALS AND METHODS

<u>Virus and Mice</u>

The murine leukemia virus stock was prepared from cell cultures of SC-1 cells (fetal mouse embryonic line) which were infected with MuLV LP-BM5. The protocol for culturing the cells and harvesting the virus are described in Suprawee Tepsuporn's senior thesis (2005).

These experiments used male BALB/c and C57BL/6 mice between the ages of seven and fifteen weeks. The mice were kept in Mount Holyoke College's animal room and were cared for in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Mice were infected intraperitoneally with 1 ml of LP-BM5 murine leukemia virus. Naïve mice which had not been exposed to the virus or any injections were also used. All mice were sacrificed by carbon-dioxide inhalation at the appropriate date post-infection. The spleens were then removed using sterile technique.

This study was approved by the Institutional Animal Care and Use Committee.

<u>Leukocyte Isolation from Spleens</u>

Leukocytes were isolated from the spleens using ficoll separation. After the spleens were removed from the animals, they were placed in a 100 μm sterile sieve in a sterile Petri dish. Two milliliters of phosphate-buffered saline (PBS) with 10% fetal bovine serum (FBS) were added to the dish and the spleen was

gently ground with the rubber end of a sterile syringe plunger in order to separate the cells from the connective tissue. The solution containing the cells was transferred to a 15 ml conical tube. The sieve and Petri dish were then washed twice with 2 ml of PBS with 10% FBS, which was added to the tube containing the cells. The 6 ml of PBS with 10% FBS containing the spleen cells were then carefully layered onto an equal volume of room temperature Sigma® Histopaque-1083 ficoll. The tubes were centrifuged at 2000 rpm for 20 minutes with the brake and acceleration off. After centrifugation, the upper layer was removed and the opaque layer containing the leukocytes was collected. The cells were then washed twice with 10 ml of PBS. The solution was centrifuged at 1800 rpm for 10 minutes for the first wash and at 1200 rpm for 10 minutes for the second wash. After the second wash, the supernatant was removed and the cells were resuspended in 10 ml of PBS with 2% FBS.

To count the cells, 10 μl of the cells were mixed with 10 μl of trypan blue and 10 μl of this solution was loaded onto a hemocytometer. Cells were centrifuged once more at 1200 rpm for 8 minutes and each sample was then resuspended in the appropriate media.

Culturing Cells for Cytokine Assays

Following leukocyte isolation from the spleen cells, approximately 5×10^6 cells from each sample were removed, centrifuged at 1200 rpm for 10 minutes, and resuspended in 500 μl of culture media. Culture media was made up in

RPMI-1640 and contained 10% heat-inactivated FBS, 100 μg/ml Penicillin-Streptomycin, and 100 μg/ml of 2 mM L-Glutamine. The cells were cultured in a 24 or 96 well plate at 37°C for 48 hours, after which the supernatant was collected and frozen at -80°C for potential future assays to evaluate cytokine concentrations.

CD8$^+$ T-Cell Isolation

CD8$^+$ T-cells were isolated from the population of spleen leukocytes through negative selection. This was done by removing all non-CD8$^+$ cells (CD4$^+$ T-cells, B cells, monocytes/macrophages, NK cells, dendritic cells, erythrocytes and granulocytes). The leukocytes were first labeled with antibodies against the non-CD8$^+$ cells. Dynabeads, which are supermagnetic polystyrene beads coated with a polyclonal sheep-anti rat IgG antibody, were added to bind to the labeled cells and a magnet was then used to separate the bead bound cells from the CD8$^+$ T-cells.

The Mouse Depletion Dynabeads used for the negative selection of CD8$^+$ T-cells were washed prior to their use. The volume of Dynabeads that would be needed (200 μl of Dyanbeads were needed for every 1 x 10^7cells) was mixed with an equal volume of Buffer 1. Buffer 1 consisted of PBS with 0.1% FBS and 2 mM EDTA. The tube containing the mixture was placed in a magnet for 1 minute, after which the supernatant was removed and discarded. The tube was

then removed from the magnet and the beads were resuspended in Buffer 1 to their initial volume.

The leukocytes collected from the spleen were resuspended in Buffer 1 so that they were at a concentration of approximately 1×10^8 cells/ml. For every 1×10^7 cells, 20 µl of heat inactivated FBS was added to the cells in Buffer 1. Next, 20 µl of Antibody Mix, which contains a mixture of rat monoclonal antibodies for mouse CD45R, CD11b, Ter-119, CD16/32 and CD4, was added for every 1×10^7 cells and the solution was incubated for 35 minutes at 4 $^{\circ}$C with occasional mixing by inversion. The cells were washed with 2 ml of Buffer 1 per 1×10^7 cells and centrifuged at 300 x g for 8 minutes in the cold. The supernatant was discarded, the cells were resuspended in 800 µl of Buffer 1 per 1×10^7 cells, and 200 µl of pre-washed Mouse Depletion Dynabeads were added per 1×10^7 cells. The solution was then incubated on a nutator for 25-30 minutes at room temperature.

After the incubation on the nutator, 1 ml of Buffer 1 was added per 1×10^7 cells and the solution was gently pipetted to resuspend the bead-bound cells. The tube was then placed in the magnet for 2 minutes. After the 2 minutes the supernatant containing the CD8$^+$ cells was collected and transferred to a new 15 ml centrifuge tube. The beads were washed twice by resuspending them in approximately 5 ml of Buffer 1 and placing the tube back in the magnet for 2 minutes. The supernatant was again collected and added to the supernatant from the first wash. The cells were centrifuged at 1200 rpm for 10 minutes and then

resuspended in 2 ml PBS with 2% FBS. There were an insufficient number of cells collected from the spleen of the 2-week infected BALB/c mouse for the CD8$^+$ isolation procedure, so CD8$^+$ cells were not isolated for that sample.

Freezing and Thawing Cells

Isolated spleen cells were centrifuged once more at 1200 rpm for 8 minutes and each sample was then resuspended in freezing media. The freezing media was consisted of 10% dimethyl sulfoxide (DMSO), 20% culture media, and 70% FBS.

For the BALB/c samples, cells were resuspended in 1 ml of freezing media and divided into two aliquots of 0.5 ml each. For the C57BL/6 samples, cells were resuspended in 2 ml of freezing media and divided into two aliquots of 1 ml each. Cells were stored at –80 ^{o}C for approximately one week and then were moved to liquid nitrogen until needed.

Cells were thawed by placing the cryogenic vials into a 37 ^{o}C water bath until all ice crystals were gone, approximately 1-2 minutes. The cells were then transferred to a 15 ml tube containing 5 ml of cold culture media and 5 ml of cold FBS and centrifuged for 8 minutes at 1200 rpm. The cells were counted and the viability of the sample was determined. They were then resuspended in 6 ml of PBS with 10% FBS and ficoll separated (see Leukocyte Isolation from Spleens). For experiments run at Amherst College, cells were not ficoll separated and were

simply resuspended in PBS with 2% FBS to a concentration of approximately 1 x 10^7 live cells/ml.

Antibody Concentrations for Flow Cytometry

These experiments used six different antibodies and two different viability dyes. Antibodies were used at a range of concentrations which differed depending on both the specific antibody and the experiment.

The allophycocyanin (APC)-labeled anti-CD8 antibodies were specific to the beta subunit of the CD8 molecule. The antibody was purchased from eBioscience along with a corresponding APC-labeled isotype control. Both were used at the concentrations recommended by the company, 2.5 µl for the anti-CD8 antibody and 11 µl for the isotype control, added directly to the 100 µl aliquots of the cells.

The APC-labeled anti-CD25 antibody was used at final concentrations ranging between 1:25 and 1:100. Final optimization tests showed a concentration of 1:25 to work best. The corresponding APC-labeled isotype control was used at a concentration of 1:200. Both antibodies were first diluted in PBS and then 10 µl of the diluted antibody was added to the 100 µl aliquots of the cells.

The Alexa Fluor® 488-labeled anti-CD8 antibody was specific to the alpha subunit of the CD8 molecule. The CD8 antibody and it's corresponding isotype control were used at a 1:10 dilution by adding 10 µl of the antibody directly to the 100 µl aliquots of the cells.

Calcein AM and CellTracker[TM] Green CMFDA (5-chloromethylfluorescein diacetate) were used as viability stains. When Calcein AM was used, 10 µl of a 1 mM solution was added to the 100 µl aliquots of the cells. The CellTracker[TM] Green came as 50 gram aliquots of dry powder. This dry powder was dissolved in 10.8 µl of DMSO. Two microliters of this solution was then diluted in 2 ml of PBS to a 10 µM concentration. One hundred microliters of the CellTracker[TM] Green was then added to the 100 µl aliquots of the cells.

Antibody Staining

The leukocytes were centrifuged at 1200 rpm for 10 minutes and resuspended in PBS with 2% FBS at a concentration of approximately 1×10^7 cells/ml, or as close to that concentration as possible if the number of cells was limited. For experiments where CellTracker[TM] Green CMFDA was used, PBS was used in all cases instead of PBS with 2% FBS to avoid possible interactions between the CellTracker[TM] Green and the proteins in the FBS.

 Each sample was divided into two 100 µl aliquots and placed into 5 ml round bottom tubes, or 1.5 ml eppendorf tubes for some of the earlier experiments. For each sample, one aliquot was stained with 10 µl of the diluted APC-labeled anti-CD25 antibody and the other aliquot was stained with 10 µl of the diluted APC-labeled rat IgG2b isotype control. Note, for the experiments on biological replicates of 2-week infected mice only one sample per strain was

stained with the APC-labeled isotype control, which was considered to be a control for all five samples. In addition, some of the samples to be run in the Amherst College flow cytometer were not stained with any APC-labeled antibodies to act as a control.

Samples were vortexed gently and incubated in the dark for 20 minutes on ice. Following the incubation, the cells were washed with 2 ml of PBS with 2% FBS, vortexed, and centrifuged at 1500 rpm for 8 minutes. The supernatant was then decanted and the cells were resuspended in 100 µl of PBS with 2% FBS. Cells were then stained with either Calcein AM or CellTracker^{TM} Green CMFDA to determine viability, or with 10 µl of a diluted Alexa Fluor® 488-labeled anti-CD8 antibody. The samples were vortexed gently and incubated in the dark for 20 minutes on ice. Cells were then washed with 2 ml PBS, vortexed, and centrifuged at 1500 rpm for 8 minutes. The supernatant was decanted and the cells were resuspended. For experiments run at Amherst College using the Beckman Coulter flow cytometer, cells were resuspended in 200 µl of PBS. For experiments using the Bioanalyzer at Mount Holyoke College, cells were resuspended in 20 µl of the cell buffer provided with the Agilent Cell Fluorescence LabChip kit. Cell assay chips were then set up according to the Agilent protocol and run in the Agilent 2100 Bioanalyzer.

To set up a chip, first 10 µl of the priming solution was added to the PS well. The chip was then allowed to sit for 60 seconds before 10 µl of the focusing dye was added to the FD well. Next, 30 µl of the cell buffer was added to each of

the CB wells. For each sample, 10 µl was added to one of the sample wells and 10 µl of cell buffer was added to any wells that did not contain a sample. The chip was then placed in the Bioanalyzer and was read with the associated Antibody Staining program.

RESULTS

CD8 Expression for Naïve and 1-week Infected Mice

Spleen cells of 1-week infected mice and naïve mice from both strains were first tested for the expression of the CD8 molecule through the use of an Allophycocyanin (APC)-labeled anti-CD8 antibody, specific to the beta subunit of the CD8 molecule. APC is a fluorescent probe which is excited at 633 nm (red) and emits at 680 nm (far red). Cells were also stained with the viability dye Calcein AM, which has an excitation wavelength of 494 nm (blue) and an emission wavelength of 517 nm (green). Calcein AM is internalized by live cells and broken down by esterases, causing the cells to fluoresce and thus allowing the live cell population to be determined. In addition, an APC-labeled isotype control antibody was used to rule out non-specific staining.

Leukocytes were collected from the spleens of naïve and 1-week infected BALB/c and C57BL/6 mice. The number of cells collected from each spleen was counted and cells were then set up at a concentration of 5×10^6 cells/ml. Cell counts show that approximately 25×10^6 cells were collected from the naïve BALB/c spleen, 16.2×10^6 cells from the naïve C57BL/6 spleen, 23.9×10^6 cells from the 1-week infected BALB/c spleen, and 15.7×10^6 cells from the 1-week infected C57BL/6 spleen. These cells were then stained and run in the Bioanalyzer.

Results from the Bioanalyzer were gated based on histograms of the fluorescence patterns for the cells. Gates were placed to separate the positively

fluorescing population from background fluorescence. Distinct peaks on the histograms differentiated the cells into positive and negative populations and the bars were placed at the level of fluorescence directly above the negative population. This was done for both the red fluorescence and blue fluorescence channels and allowed for determination of the percentage of cells counted by the Bioanalyzer that positively fluoresced both red and green (Table 1). This percentage should then indicate the percent of live cells that were expressing the molecule for which the APC-labeled antibody was specific. Figures 1 and 2 show scatter plots of the fluorescence patterns for the BALB/c and C57BL/6 mice. Unexpectedly, the control showed equal or higher percentages of gated cells in all except one case making it difficult to draw conclusions about the reliability of the gated percentages for the CD8 antibody (Figure 3).

This experiment was run twice to verify the results. Each run used one BALB/c and one C57BL/6 mouse at one week post-infection, and one naïve mouse of each strain. Results from the second experiment confirmed results from the first and showed the same patterns for the gated cells.

Table 1. Bioanalyzer event counts and percent of gated cells expressing both Calcein and APC-labeled anti-CD8 antibody fluorescence for naïve and 1-week infected BALB/c and C57BL/6 mice.

Mouse and infection time point	Anti-CD8β antibody		Isotype Control	
	Number of Events*	Percent Gated	Number of Events*	Percent Gated
BALB/c naïve	118	3.9	731	15.8
C57BL/6 naïve	107	8.3	1934	8.2
BALB/c 1-week	257	10.3	734	12.4
C57BL/6 1-week	3078	18.3	2043	8.0

* One event is one cell counted by the Bioanalyzer
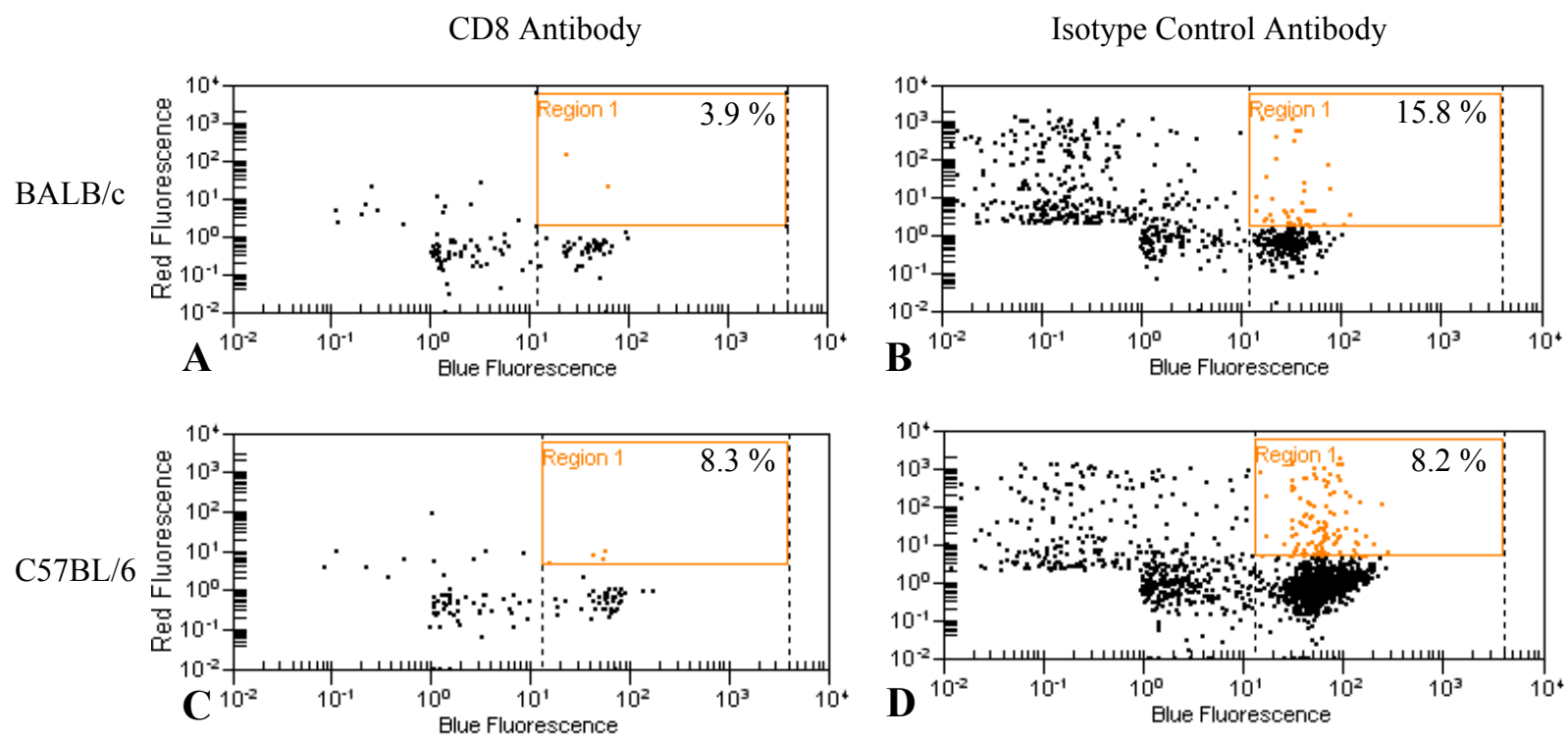
Figure 1. Spleen leukocytes from naive BALB/c and C57BL/6 mice stained with the CD8 antibody or corresponding control antibody. (A) BALB/c cells labeled-with APC-labeled CD8β antibody (red) and Calcein AM (blue), (B) BALB/c cells labeled with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 cells labeled with APC-labeled CD8β antibody and Calcein AM, (D) C57BL/6 cells labeled with APC-labeled isotype control antibody and Calcein AM.
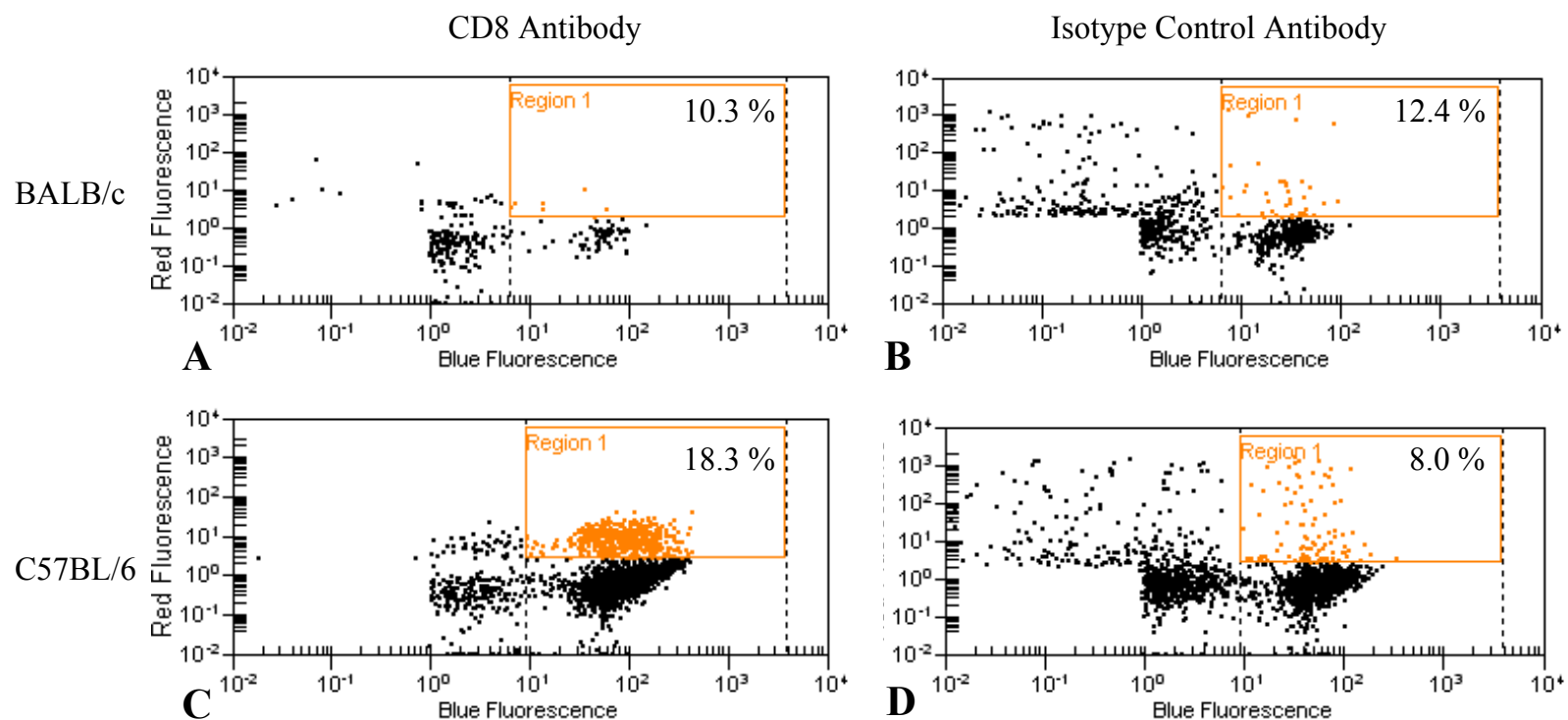
Figure 2. Spleen leukocytes from 1 week infected BALB/c and C57BL/6 mice stained with the CD8 antibody or corresponding control antibody. (A) BALB/c cells labeled-with APC-labeled CD8β antibody (red) and Calcein AM (blue), (B) BALB/c cells labeled with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 cells labeled with APC-labeled CD8β antibody and Calcein AM, (D) C57BL/6 cells labeled with APC-labeled isotype control antibody and Calcein AM.
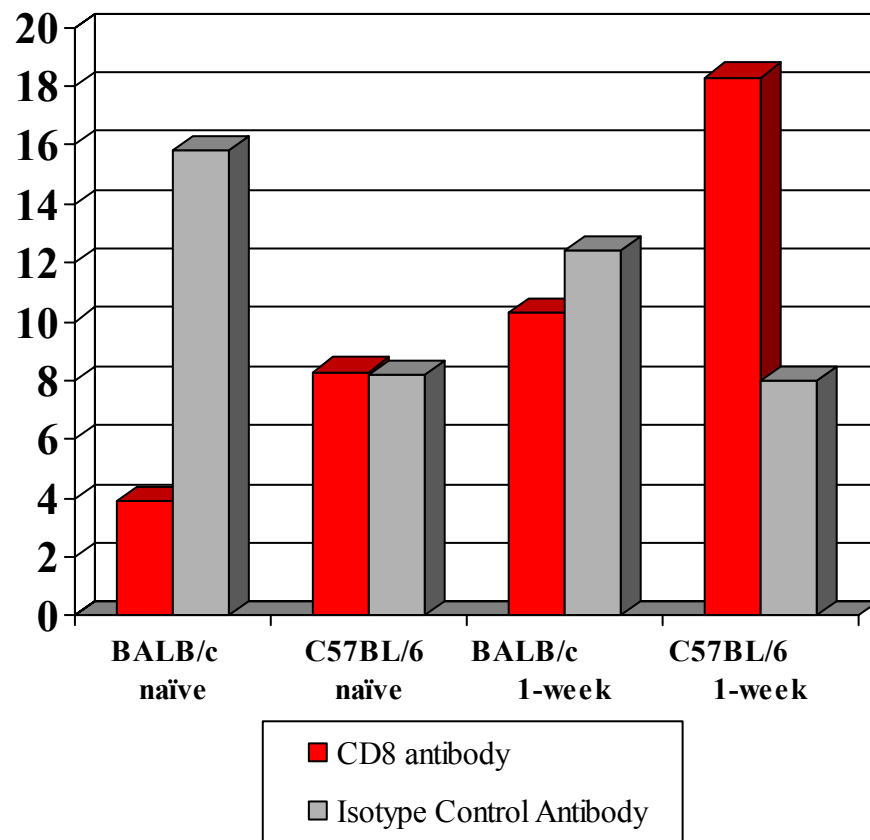
Figure 3. Percent of live cells labeled with the APC antibody for naïve and 1-week infected BALB/c and C57BL/6 mice.

Antibody Optimizations for APC-labeled anti-IL-2Rα, Control Antibodies, and CellTracker[TM] Green

The APC-labeled anti-CD25 antibody and APC-labeled rat IgG2b isotype control antibody were tested at varying concentrations to optimize their performance. Naïve BALB/c spleen leukocytes were used for this experiment. The recommended concentration for the anti-CD25 antibody was given as 0.3 µg per 10^6 cells (and came at a concentration of 0.1 µg/µl). Since earlier experiments used approximately 5 x 10^5 cells, the antibody was originally tested at concentrations of 1:50, 1:75, and 1:100. The IgG2b isotype control antibody had a recommended concentration of 1:100, so it was tested at concentrations of 1:100, 1:150, and 1:200. Based on the results, the APC-labeled anti-CD25 antibody was determined to work best at a 1:75 concentration and the APC-labeled rat IgG2b isotype control antibody was determined to work best at a 1:200 concentration (results not shown).

In later experiments only low levels of staining were being observed, so the APC-labeled anti-CD25 antibody was optimized a second time, this time for 10^6 cells. Thawed spleen cells from a 3-day infected C57BL/6 mouse were used for this experiment. The antibody was tested at concentrations of 1:30, 1:50, and 1:75, and 1:30 was determined to work the best (results not shown). Thus for the final experiments, on biological replicates at the 2-week infected time point, the APC-labeled anti-CD25 antibody was used at a 1:25 concentration.

Other changes that were made to improve results from the Bioanalyzer included starting the staining protocol with 10^6 cells instead of 5 x 10^5 cells, still

in 100 µl aliquots, to increase event counts. Also, 1.5 ml eppendorf tubes were replaced with 5 ml round bottom tubes to optimize the staining process.

To determine the best concentration for the CellTracker™ Green, it was tested at final concentrations of 2.5 µM, 5 µM, and 10 µM. There appeared to be no noticeable difference in the effectiveness of the dye between the three concentrations, so it was used at 5 µM for all experiments (results not shown). Due to difficulties with some of the initial tests, PBS was used during the staining protocol instead of PBS with 2% FBS to avoid possible interactions with the proteins in FBS.

CD8$^+$ T-Cell Isolation and IL-2R Experiments

To observe the expression of the high affinity interleukin-2 receptor (IL-2R) on CD8$^+$ T-cells, an APC-labeled anti-CD25 (IL-2Rα) antibody was used to stain isolated CD8$^+$ leukocytes. The APC-labeled anti-CD25 antibody and corresponding APC-labeled rat IgG2b isotype control antibody were purchased from SouthernBiotech. CD8$^+$ cells were isolated using the Dynal® Mouse CD8 Negative Isolation Kit from Dynal® Biotech.

Leukocytes were collected from the spleens of naïve, 1-week, 2-week, and 4-week infected BALB/c and C57BL/6 mice. A negative isolation procedure was then used to collect only the CD8$^+$ cells. For each spleen the total number of leukocytes collected was calculated as well as the number of CD8$^+$ cells that were able to be isolated (Table 2).

Table 2. Total number of live leukocytes and number of CD8$^+$ cells collected from naïve, 1-week, 2-week, and 4-week infected BALB/c and C57BL/6 spleens.

	BALB/c total live leukocytes	BALB/c CD8$^+$ cells	C57BL/6 total live leukocytes	C57BL/6 CD8$^+$ cells
Naïve	18.8 x 10^6	3.2 x 10^5	26.8 x 10^6	4.4 x 10^5
1-week post-infection	38.2 x 10^6	4.8 x 10^5	39.6 x 10^6	10.4 x 10^5
2-weeks post-infection	10.8 x 10^6	n/a	64.4 x 10^6	12.4 x 10^5
4-weeks post-infection	19.2 x 10^6	2.0 x 10^5	58.0 x 10^6	10.0 x 10^5

Once the CD8$^+$ cells were isolated, they were stained with both APC-labeled anti-CD25 antibodies and Calcein AM (viability dye) and run on the Bioanalyzer. Red fluorescence showed the cells that are expressing CD25 and the Calcein staining showed the live cells. Populations that were positive for both Calcein and APC fluorescence were gated using the same methods as described for the CD8 antibody experiments. The raw data of the number of events counted and percent of gated events for each sample is shown in Table 3 and scatter plots of the fluorescence patterns for each time point can be seen in Figures 4 -7. Each event represents one cell counted by the Bioanalyzer.

To account for non-specific binding, the percent of gated events for the isotype control was subtracted from the percent of gated events for the anti-CD25 antibody. From this it appears that for both naïve mice the percent of CD8$^+$ cells expressing CD25 is zero. At one week after infection, the BALB/c mouse increases to 7.5 percent of the CD8$^+$ cells expressing CD25 while the C57BL/6 mouse only shows 0.2 percent of the CD8$^+$ cells expressing CD25. However, at 2 weeks after infection the C57BL/6 mouse shows 26.1 percent of the CD8$^+$ cells expressing CD25. There were not enough CD8$^+$ cells from the 2 week infected BALB/c mouse, so no data are available for that strain at that time point. At four weeks, the BALB/c mouse has returned to zero percent of the CD8$^+$ cells expressing CD25, but the C57BL/6 mouse still shows 19.5 percent of the CD8$^+$ cells expressing CD25. (Figure 8)

Table 3. CD25 expression in isolated CD8$^+$ cells from spleens of BALB/c and C57BL/6 mice.

	Anti-CD25 antibody		Isotype Control	
Mouse	Number of Events*	Percent Gated	Number of Events*	Percent Gated
BALB/c naïve	571	3.2	811	3.4
C57BL/6 naïve	2794	2.3	2866	5.6
BALB/c 1-week,	423	7.5	36	0
C57BL/6 1-week,	935	3.4	1559	3.2
BALB/c 2-week	n/a	n/a	n/a	n/a
C57BL/6 2-week,	1038	27.1	1335	1.0
BALB/c 4-week,	403	4.7	717	12.9
C57BL/6 4-week,	528	21.3	1024	1.8

* One event is one cell counted by the Bioanalyzer

Figure 4. Isolated CD8^{+} cells from the spleens of a naive BALB/c mouse and a naïve C57BL/6 mouse stained with the CD25 antibody or corresponding control antibody. (A) BALB/c CD8^{+} cells stained with APC-labeled anti-CD25 antibody (red) and Calcein AM (blue), (B) BALB/c CD8^{+} cells stained with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 CD8^{+} cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (D) C57BL/6 CD8^{+} cells stained with APC-labeled isotype control antibody and Calcein AM.
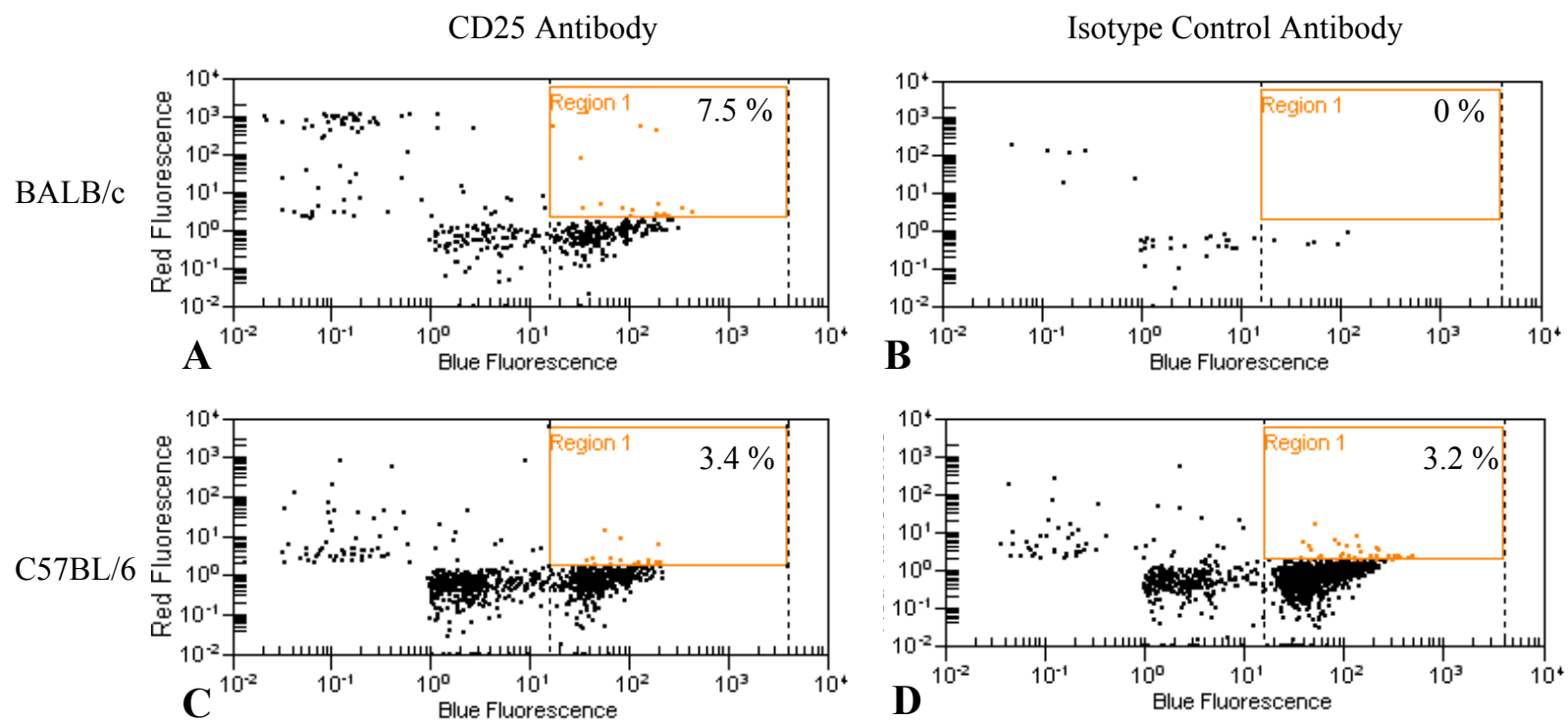
Figure 5. Isolated CD8^{+} cells from the spleens of a 1-week infected BALB/c mouse and a 1-week infected C57BL/6 mouse stained with the CD25 antibody or corresponding control antibody. (A) BALB/c CD8^{+} cells stained with APC-labeled anti-CD25 antibody (red) and Calcein AM (blue), (B) BALB/c CD8^{+} cells stained with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 CD8^{+} cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (D) C57BL/6 CD8^{+} cells stained with APC-labeled isotype control antibody and Calcein AM.
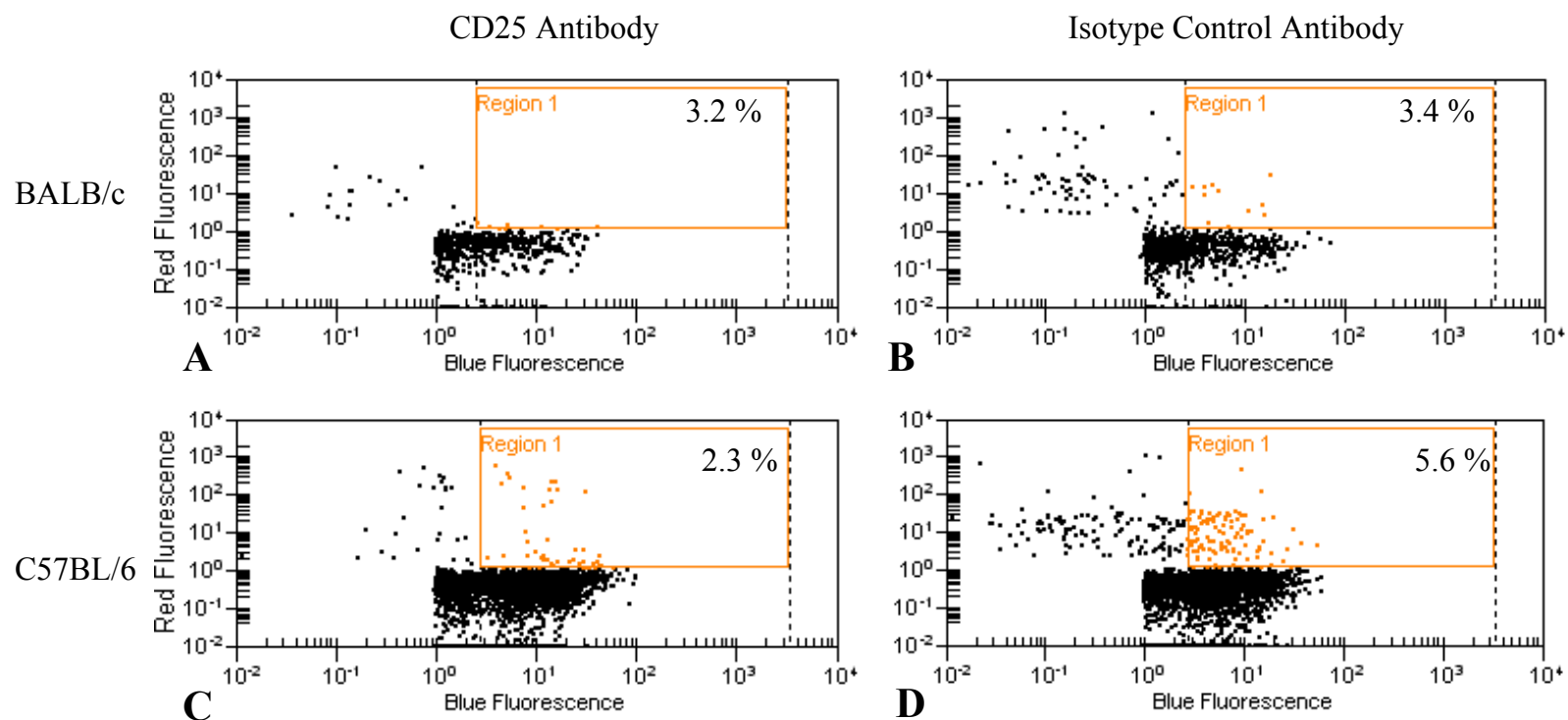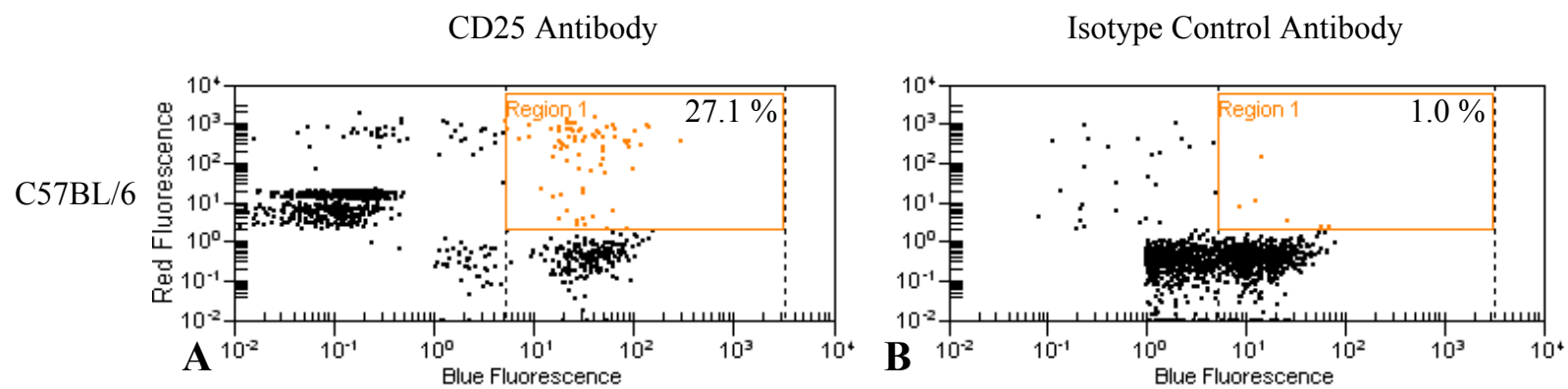
Figure 6. Isolated CD8^{+} cells from the spleens of a 2-week infected C57BL/6 mouse stained with the CD25 antibody or corresponding control antibody. (A) C57BL/6 CD8^{+} cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (B) C57BL/6 CD8^{+} cells stained with APC-labeled isotype control antibody and Calcein AM.

Figure 7. Isolated CD8$^+$ cells from the spleens of a 4-week infected BALB/c mouse and a 4-week infected C57BL/6 mouse stained with the CD25 antibody or corresponding control antibody. (A) BALB/c CD8$^+$ cells stained with APC-labeled anti-CD25 antibody (red) and Calcein AM (blue), (B) BALB/c CD8$^+$ cells stained with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 CD8$^+$ cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (D) C57BL/6 CD8$^+$ cells stained with APC-labeled isotype control antibody and Calcein AM.
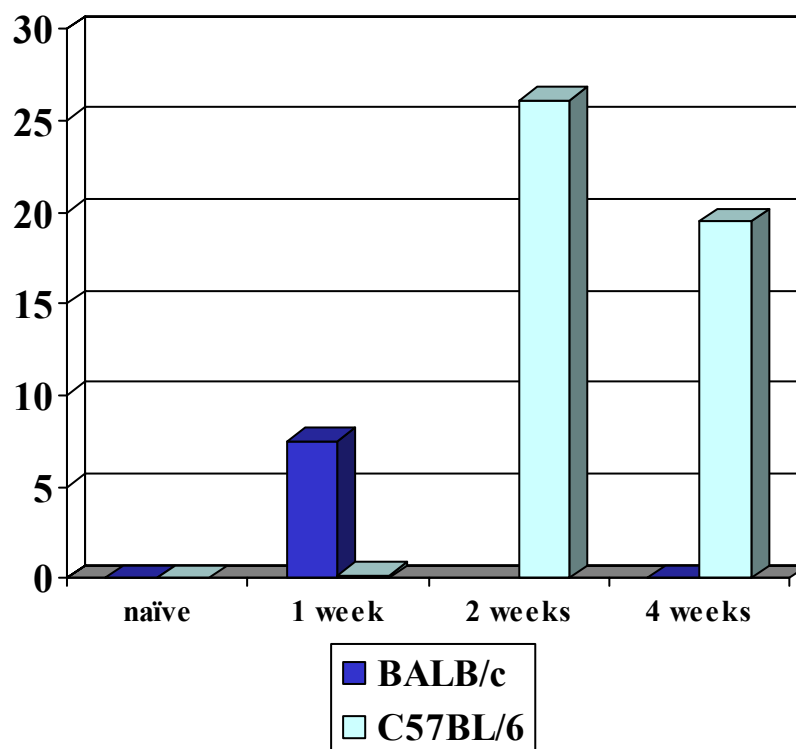
Figure 8. Percent of CD8$^+$ cells expressing CD25 for naïve, 1-week, 2-week and 4-week infected BALB/c and C57BL/6 mice. (Percents have been adjusted by subtracting the percent of gated cells for the corresponding isotype control from the percent of gated cells for the sample stained with the anti-CD25 antibody)

The percent of unseparated leukocytes expressing CD25 was also determined for naïve and 2-week infected BALB/c and C57BL/6 mice. Figures 9 and 10 show the scatter plots of the fluorescence patterns. When percentages were adjusted by subtracting the percent of gated cells in the control from the percent of gated cells in the sample stained with the APC-labeled anti-CD25 antibody, the naïve BALB/c mouse had 27.9 percent of the cells expressing CD25 and the naïve C57BL/6 mouse had 13.4 percent of the cells expressing CD25. At 2-weeks post-infection, BALB/c had 28.1 percent of the cells expressing CD25 and C57BL/6 had 30.8 percent of the cells expressing CD25. Raw data of the number of events and percent of gated cells is presented in Table 4.

Table 4. CD25 expression in unseparated spleen leukocytes from BALB/c and C57BL/6 mice.

Mouse		Anti-CD25 antibody		Isotype Control	
		Number of Events*	Percent Gated	Number of Events*	Percent Gated
BALB/c	naïve	2671	34.4	3201	6.5
C57BL/6	naive	2581	17.5	3193	4.1
BALB/c	2-week	3743	33.3	2581	5.2
C57BL/6	2-week	2022	34.4	2812	3.6

* One event is one cell counted by the Bioanalyzer
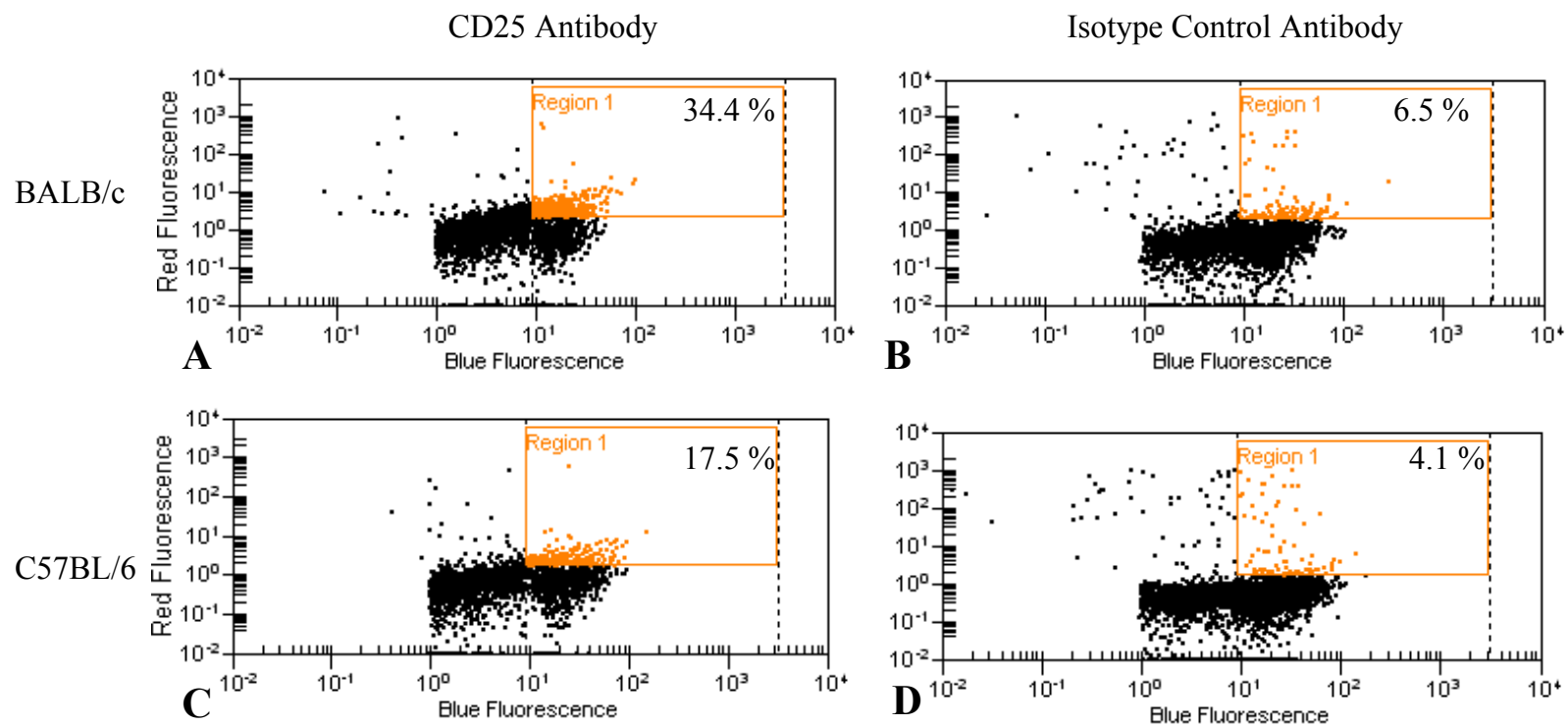
Figure 9. Leukocytes from the spleen of a naive BALB/c mouse and a naïve C57BL/6 mouse stained with the CD25 antibody or corresponding control antibody. (A) BALB/c cells stained with APC-labeled anti-CD25 antibody (red) and Calcein AM (blue), (B) BALB/c cells stained with A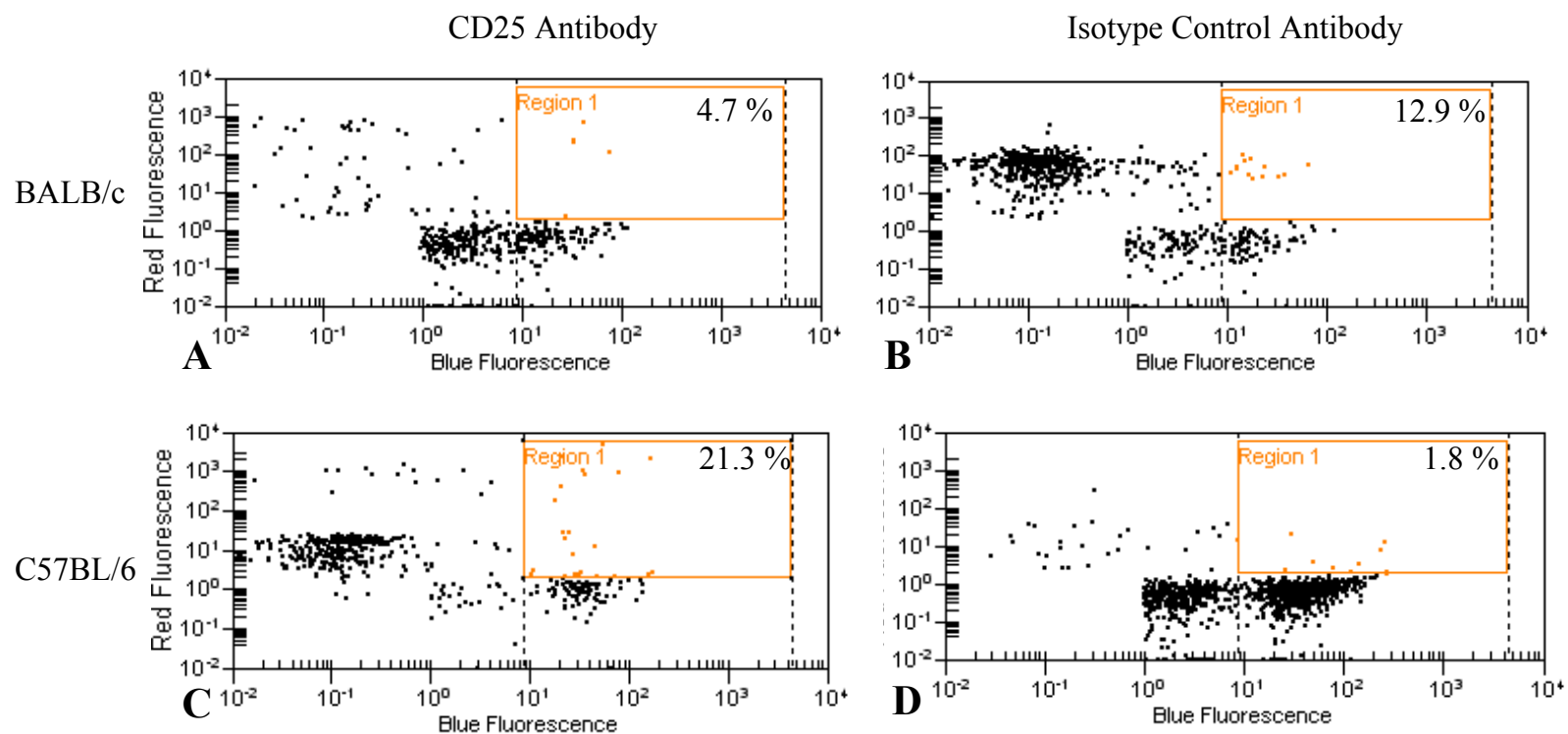PC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (D) C57BL/6 cells stained with APC-labeled isotype control antibody and Calcein AM.
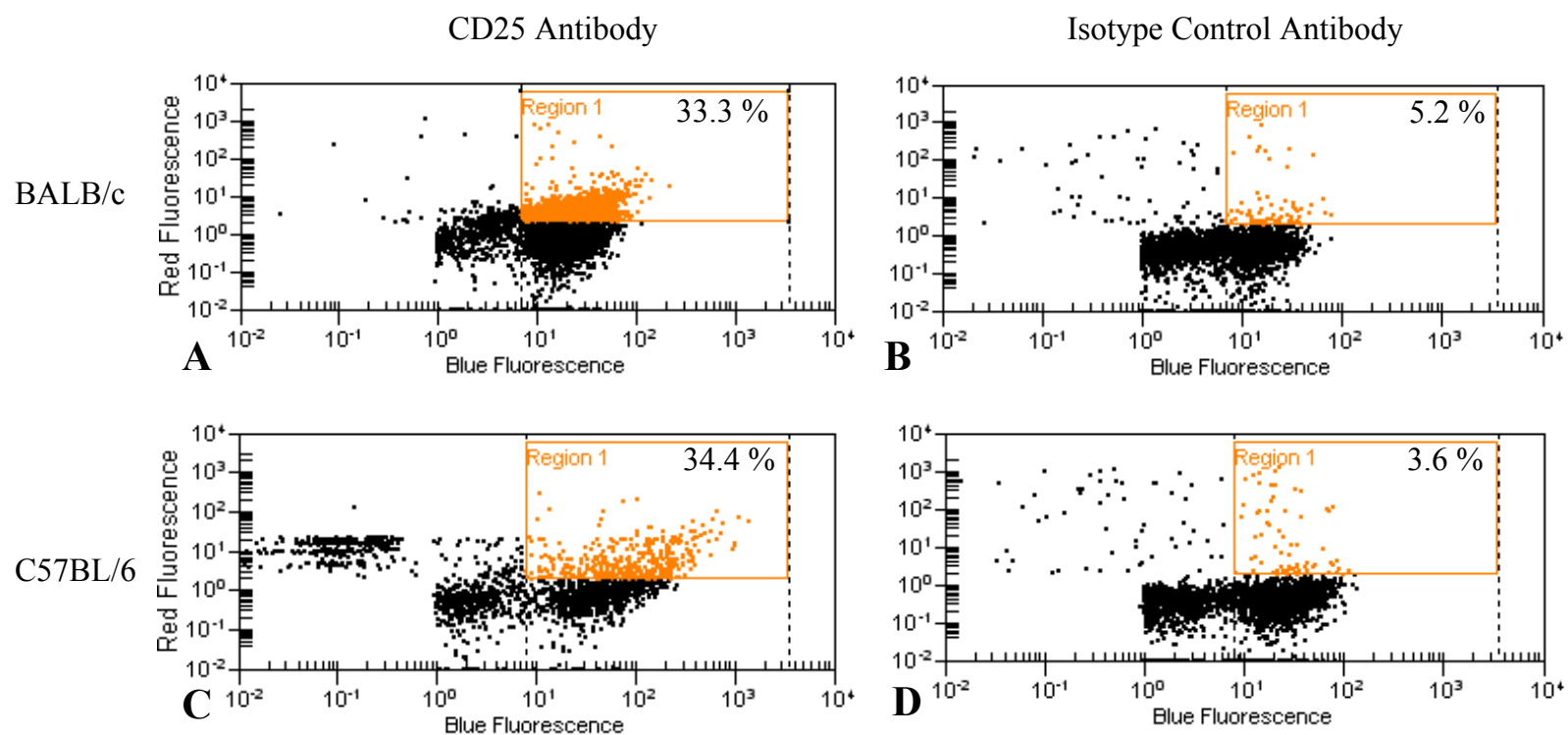
Figure 10. Leukocytes from the spleens of 2-week infected BALB/c and C57BL/6 mice stained with the CD25 antibody or corresponding control antibody. (A) BALB/c cells stained with APC-labeled anti-CD25 antibody (red) and Calcein AM (blue), (B) BALB/c cells stained with APC-labeled isotype control antibody and Calcein AM, (C) C57BL/6 cells stained with APC-labeled anti-CD25 antibody and Calcein AM, (D) C57BL/6 cells stained with APC-labeled isotype control antibody and Calcein AM.

Flow Cytometry Using Antibodies to CD8 and CD25

To address some of the problems associated with isolation of the CD8$^+$ cells, I tried double antibody staining with antibodies to both the CD8 molecule (alpha subunit) and CD25. Thus, the cells which show both red and green fluorescence would be both CD8$^+$ and CD25$^+$. The APC-labeled anti-CD25 antibody was the same as prior experiments. An Alexa Fluor® 488 anti-CD8α antibody and corresponding Alexa Fluor® 488 rat IgG2a negative control antibody were purchased from Serotec. Alexa Fluor® 488 has an excitation wavelength of 495 nm and an emission wavelength of 519 nm (green).

Intitial tests showed that the Agilent 2100 Bioanalyzer was unable to detect the Alexa Fluor® 488 antibody (results not shown). Therefore, samples were taken to Amherst College to be run in their flow cytometer. The protocol was first tested to verify that the antibodies would work and to determine the proper concentrations for their use in the flow cytometer. These initial experiments were run on cells that had been frozen during the previous semester's immunology class. Each antibody was tested at two different concentrations and was tested alone, as well as combinations of the APC-labeled and Alexa Fluor® 488 antibodies together (see Table 5). In addition, one of the samples of cells was left unstained. Each sample was run in the flow cytometer and the resulting data was analyzed using WinMDI 2.8. Due to the high gating of the red channel during the first practice experiment, a second experiment was run to verify results.

The list file created by the flow cytometer was opened using WinMDI and a scatter plot of forward scatter vs. side scatter was generated for each sample. Based on the forward and side scatter distribution of the cells a region was selected which was determined to contain the population of live cells. Histograms were then generated for both red and green fluorescence of this selected population. Figure 11 shows histograms of the green fluorescence for samples stained with the Alexa Fluor® 488 CD8-antibody only, the APC CD25-antibody only, both antibodies together, and isotype controls for both Alexa Fluor® 488 and APC. The peak of bright fluorescence that only appears for the samples stained with the Alexa Fluor® 488 CD8-antibody is the population of $CD8^+$ cells. Figure 12 shows histograms of the red fluorescence for the same samples as shown in Figure 11. However, no peak of bright fluorescence can be seen in the samples stained with the APC CD25-antibody. Therefore it appears that the APC antibody was not being detected by the flow cytometer.

Table 5. Antibodies added to each sample for initial tests using the Amherst flow cytometer.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
APC-CD25	a	b	-	-	a	b	-	-	-	-	-	-	-
Alexa-CD8α	-	-	d	e	d	e	-	-	-	-	-	-	-
APC control	-	-	-	-	-	-	b	c	-	-	b	c	-
Alexa control	-	-	-	-	-	-	-	-	d	e	d	e	-

a = 1:75 dilution, b = 1:100 dilution, c = 1:200 dilution, d = 1:5 dilution,

e =   1:10 dilution, a dash indicates the sample was not stained with that antibody
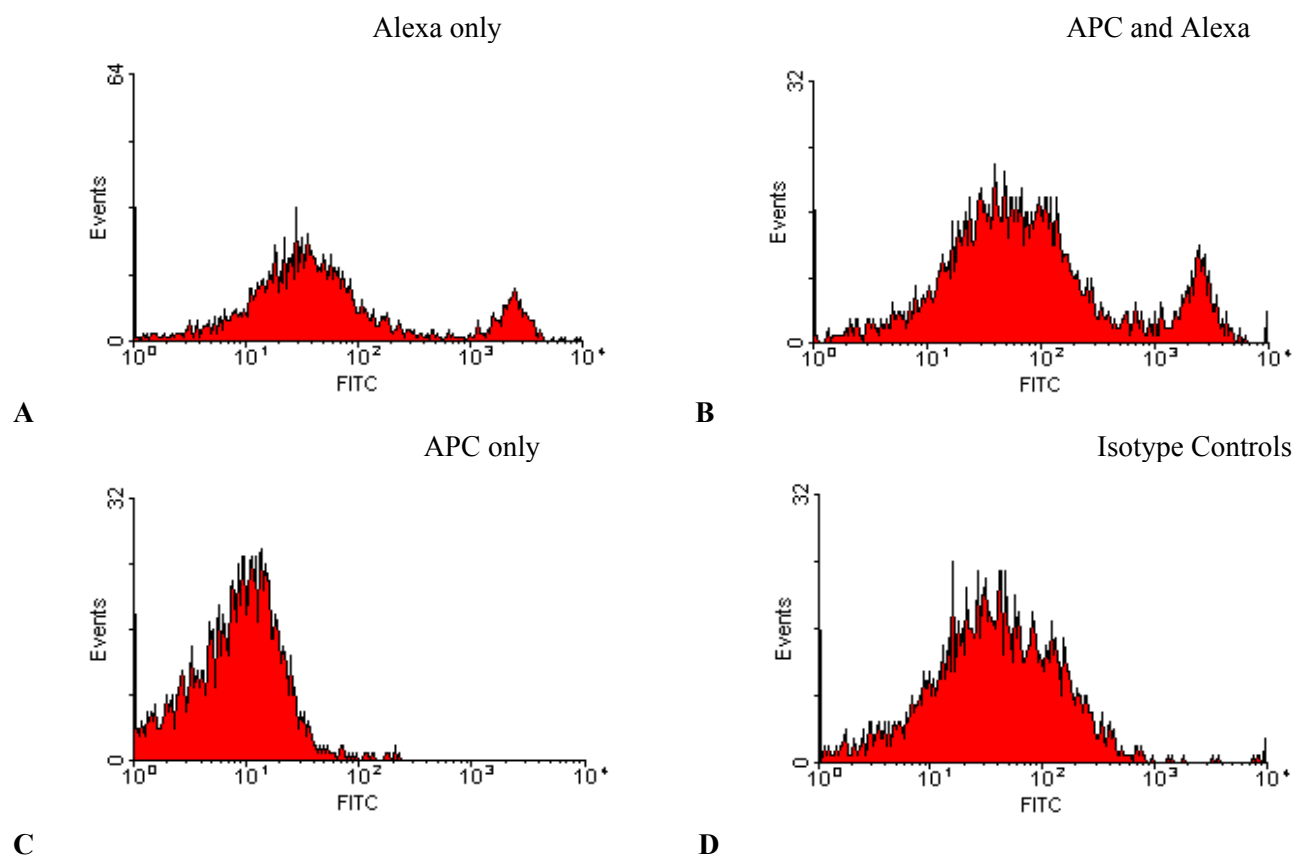
Figure 11. Histogram of green fluorescence for thawed spleen cells stained with A) Alexa Fluor® 488-labeled anti-CD8alpha antibody at a 1:10 dilution, B) Alexa Fluor® 488-labeled anti-CD8alpha antibody at a 1:10 dilution and APC-labeled anti-CD25 antibody at a 1:100 dilution, C) APC-labeled anti-CD25 antibody at a 1:100 dilution, D) Alexa Fluor® 488-labeled isotype control at a 1:10 dilution and APC-labeled isotype control at a 1:200 dilution.
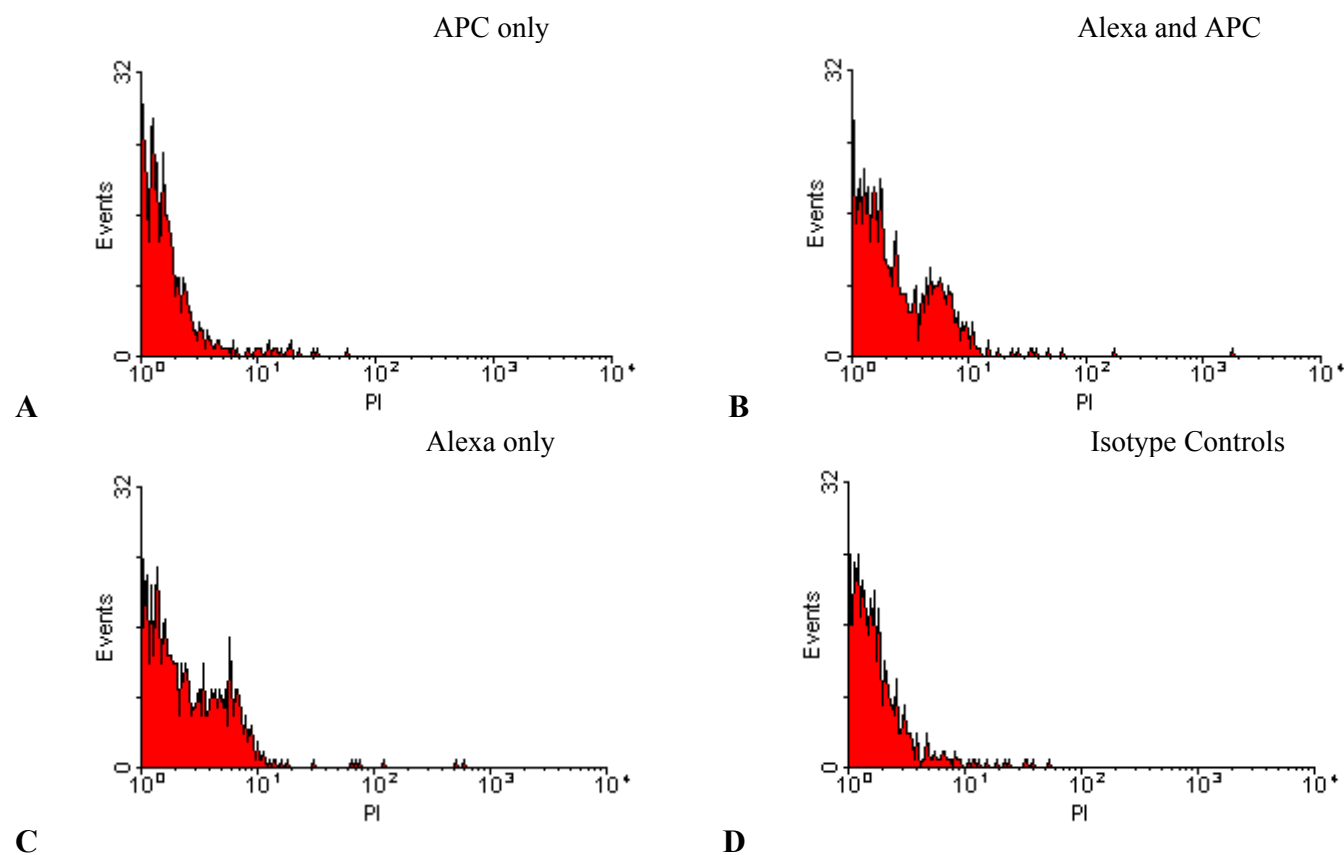
Figure 12. Histogram of red fluorescence for thawed spleen cells stained with A) APC-labeled anti-CD25 antibody at a 1:100 dilution, B) Alexa Fluor® 488-labeled anti-CD8α antibody at a 1:10 dilution and APC-labeled anti-CD25 antibody at a 1:100 dilution, C) Alexa Fluor® 488-labeled anti-CD8α antibody at a 1:10 dilution, D) Alexa Fluor® 488-labeled isotype control at a 1:10 dilution and APC-labeled isotype control at a 1:200 dilution.

CD25 Expression on Unseparated Leukocytes from 2-week Infected Mice

Based on the results from the initial CD8$^+$ isolation and IL-2R experiments I decided to focus on the 2 week time point and used biological replicates to confirm the preliminary results which had come from only one mouse of each strain. However, due to the fact that the collected leukocytes from the 2 week infected mice had to be frozen, the CD8$^+$ isolation procedure was no longer feasible and the flow cytometer at Amherst was unable to detect the APC-labeled anti-CD25 antibody. Therefore I looked at the expression of CD25 on all leukocytes from the spleen instead of CD8$^+$ cells only.

After the cells were thawed they were ficoll separated to remove the dead cells and increase the viability of the sample. Following the ficoll separation the number of cells was counted and then the cells were resuspended to a concentration of 1 x 10^7 cells/ ml (Table 6).

Table 6. Number of live leukocytes, after thawing and ficoll separation, for 5 biological replicates of 2-week infected BALB/c and C57BL/6 mice.

	BALB/c	C57BL/6
1	1.9×10^6	4.4×10^6
2	1.6×10^6	2.8×10^6
3	1.3×10^6	2.8×10^6
4	1.0×10^6	1.6×10^6
5	0.5×10^6	2.0×10^6

The leukocytes were then stained with the APC-labeled anti-CD25 antibody and CellTracker[TM] Green (viability dye) and run in the Bioanalyzer. Like Calcein, CellTracker[TM] Green is a viability dye that is cleaved by esterases in the cytoplasm of a cell creating a fluorescent product with an excitation wavelength of 492 nm and an emission wavelength of 517 nm (green).

Cells were gated using the histogram of the isotype control for each mouse strain and the gate was kept the same for all samples within a strain. Raw data of the number of events and percent of gated cells is presented in Table 7. Figures 13 and 14 show scatter plots of the fluorescence patterns for each sample. The C57BL/6 mice show higher expression of the CD25 molecule on spleen leukocytes at 2 weeks after infection than the BALB/c mice. On average the C57BL/6 mice had 35.4 percent (SD ± 13.1) of the spleen leukocytes expressing CD25, while the BALB/c mice had 11.9 percent (SD ± 5.2) of the spleen leukocytes expressing CD25 (Figure 15). This difference was determined to be statistically significant ($p < 0.01$)

Table 7. CD25 expression in unseparated spleen leukocytes from 2-week infected BALB/c and C57BL/6 mice.

	BAL B/c		C57BL/6	
Sample	Number of Events*	Percent Gated	Number of Events*	Percent Gated
1	1047	11.4	1646	25.2
2	2738	21.2	1379	36.4
3	2068	18.1	1190	26.5
4	1169	10.5	1752	61.6
5	464	7.0	1151	38.9
Isotype Control	715	1.7	1824	2.3

* One event is one cell counted by the Bioanalyzer
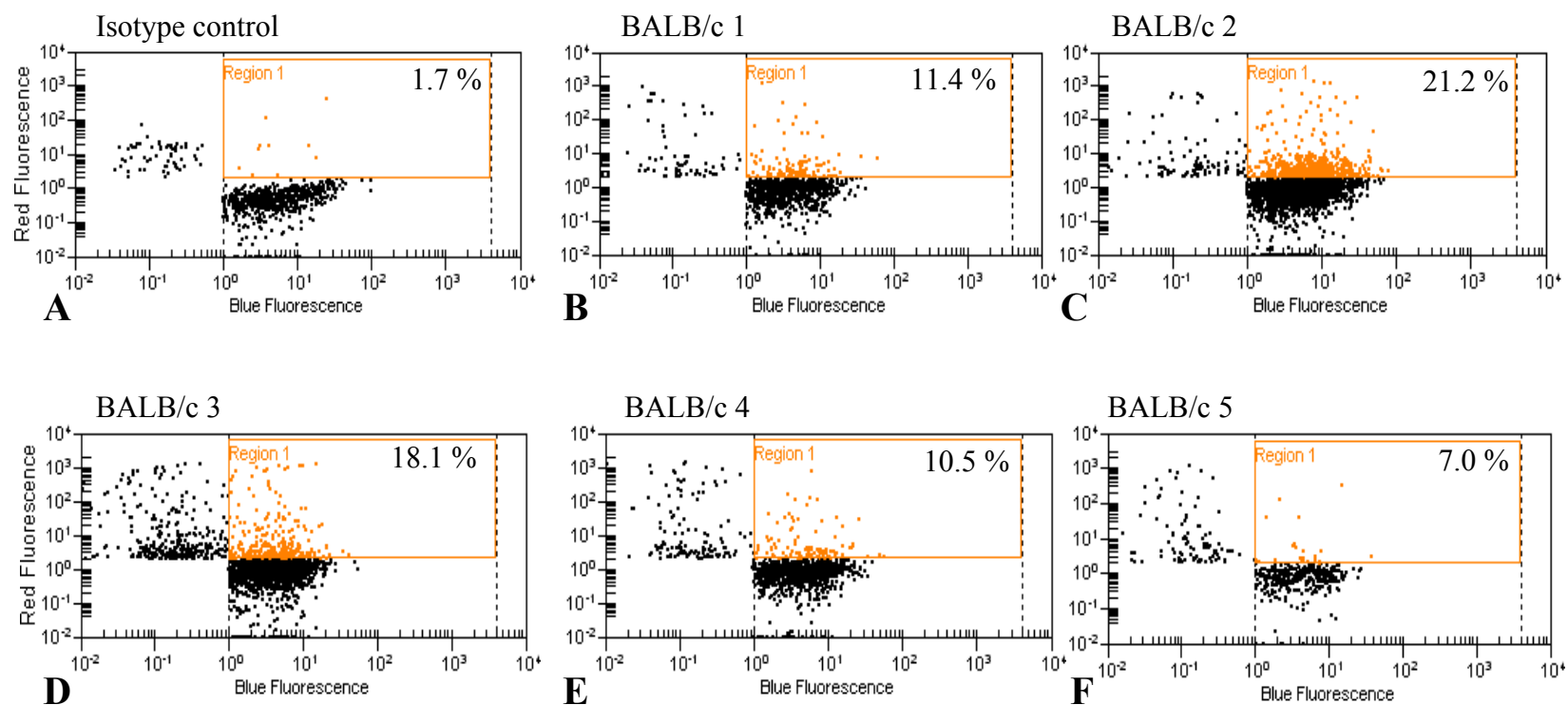
Figure 13. CD25 expression on spleen leukocytes from 2-week infected BALB/c mice. (A) stained with APC-labeled isotype control antibody (red) and CellTracker[TM] Green (blue), (B) - (F) biological replicates stained with APC-labeled anti-CD25 antibody and CellTracker[TM] Green.
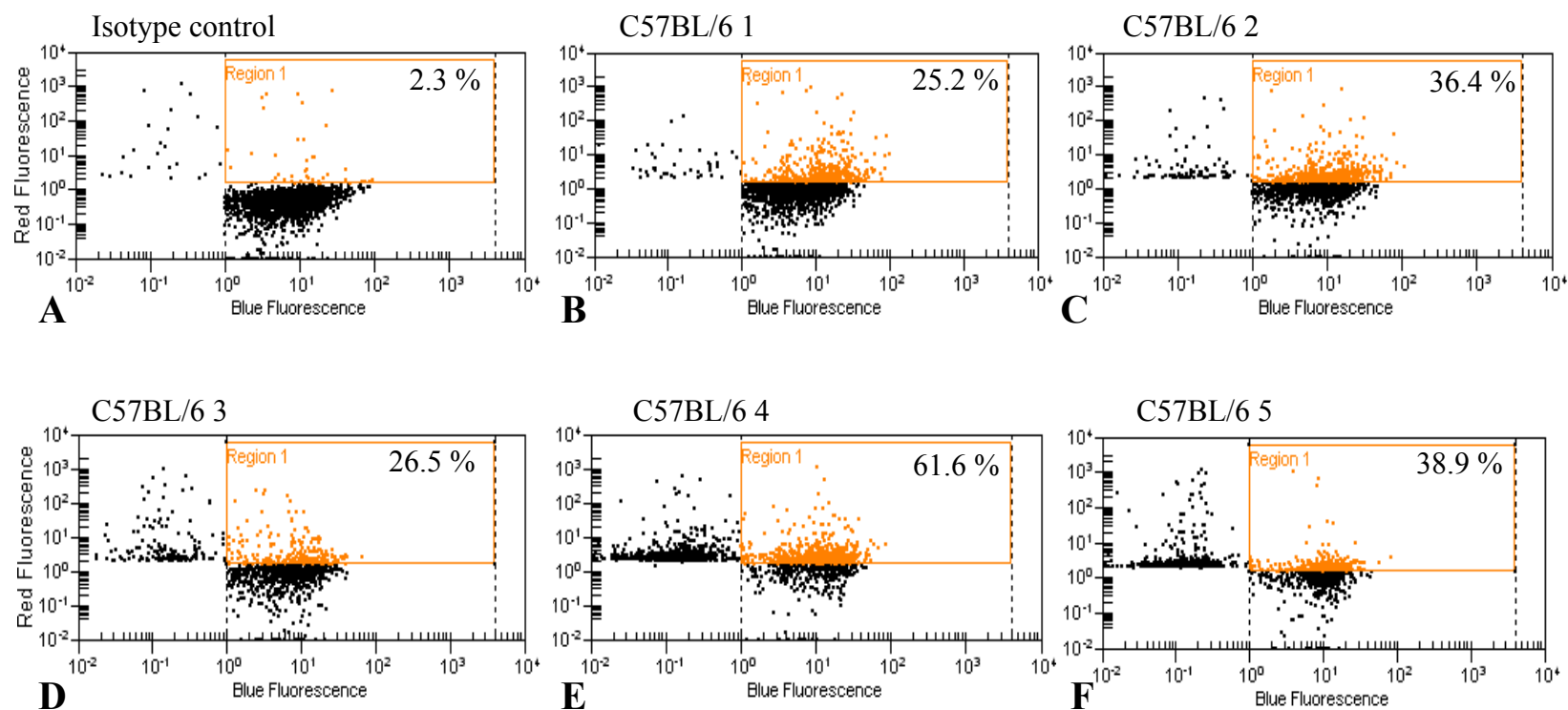
Figure 14. CD25 expression on spleen leukocytes from 2-week infected C57BL/6 mice. (A) stained with APC-labeled isotype control antibody (red) and CellTracker™ Green (blue), (B) - (F) biological replicates stained with APC-labeled anti-CD25 antibody and CellTracker™ Green.
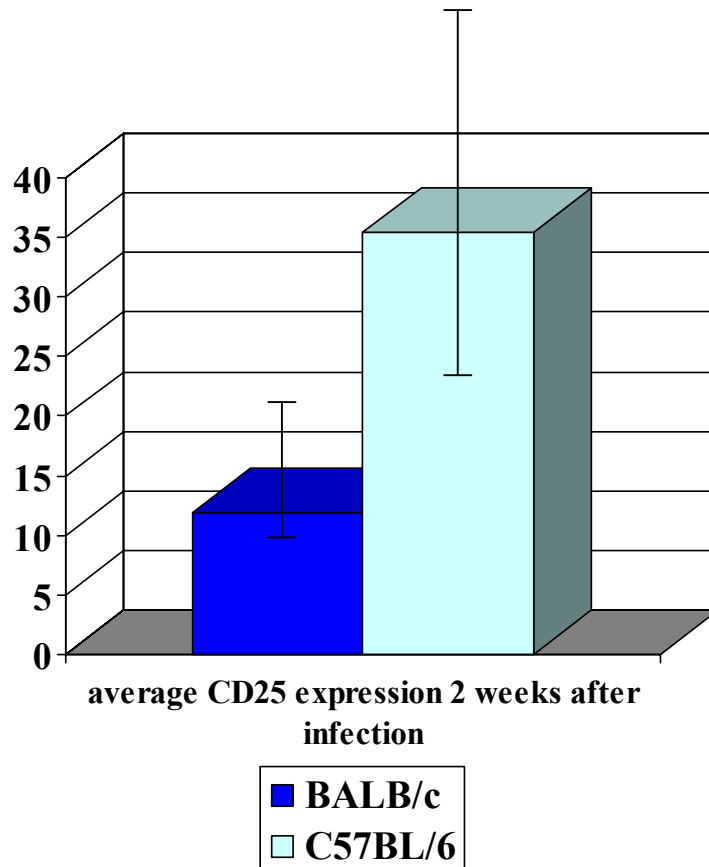
Figure 15. Average CD25 expression on spleen leukocytes for 5 biological replicates of BALB/c mice and 5 biological replicates of C57BL/6 mice. The standard deviation for BALB/c is ± 14.6 and the standard deviation for C57BL/6 is ± 5.8. The difference between the two strains is statistically significant ($p < 0.01$).

DISCUSSION

This study originally set out to investigate the expression of the high affinity IL-2 receptor on CD8^{+} T-cells in hopes of learning more about the potential of these cells to proliferate, respond to antigens, and perform their cytotoxic effector functions. This was done by isolating CD8^{+} T-cells and staining with a fluorescent antibody to the IL-2R alpha subunit (CD25), as well staining leukocytes with fluorescent antibodies to both CD8 and CD25. However, neither method was able to provide conclusive results. Nonetheless, I was able to show that there is a statistically significant difference in the percent of spleen leukocytes expressing CD25 between 2-week infected BALB/c and C57BL/6 mice.

Problems Encountered

Throughout the course of this study I encountered various difficulties with my experiments including the number of leukocytes collected from the spleen, the number of CD8^{+} T-cells able to be isolated, low event counts from the Bioanalyzer, and difficulties with antibody staining.

To increase the number of leukocytes I could collect from the spleen, I tried various buffers during the ficoll separation. These included RPMI with 10% FBS, PBS with 2% FBS, and PBS with 10% FBS. Using PBS with 10% FBS appeared to work the best, followed by two washes with PBS. To increase the number of CD8^{+} T-cells isolated during the negative selection protocol,

I extended the incubation time for both the Antibody Mix and Dynabeads. In addition, I added two washes after the beads were first separated and the supernatant containing the CD8$^+$ cells was collected. Regarding the viability of thawed cells, the most important components of the freezing and thawing procedures appear to be quickly placing the cells at -80 ^{o}C, moving them to liquid nitrogen within a relatively short period of time, and quickly transferring them to cold media once they are thawed.

I also attempted to address the problems related to the antibody staining and event counts. I switched from using 1.5 ml eppendorf tubes to 5 ml round bottom tubes for the staining, and increased the number of cells I started with from 5×10^5 to 1×10^6. These changes appear to have improved the results of the flow cytometry, as my final experiments had no event counts below 500, and most were well above 1000.

I also tested the antibodies at various concentrations to optimize their performance. Since I had to go through two optimization tests, it would appear that the best strategy is to test the antibody and control antibody at a number of concentrations above and below the company's recommendation. Each chip has the space for six samples, allowing for the control antibody to be tested at up to six concentrations and the positive antibody to be tested at five different concentrations. It also appears that testing for only red fluorescence, without also staining with a viability stain such as Calcein or CellTracker^{TM} Green does not provide much useful information.

CD8 Expression

The purpose of the original experiment looking at CD8 expression was two-fold. First, I had hoped to confirm that the decrease in the percent of CD8$^+$ T-cells which is cited to occur in susceptible mice can been seen at earlier time points than the ones discussed in the literature (Morse *et al.*, 1989). I had also wanted to show how the percentage of CD8$^+$ T-cells behaves in BALB/c mice, since the literature focuses on the susceptible strains. Unfortunately, the control antibody that was purchased with the APC-labeled anti-CD8 antibody did not work as expected. In almost every case there was a higher percentage of staining for the control than for the CD8 antibody. Not only was the percent of cells stained by the control uncharacteristically high, but the level of fluorescence was also extremely high, with intensity levels up to 10^3. The amount of control antibody to be used, as recommended by the company, was also much higher than typically seen for antibody staining. After the first set of results, I considered that I might have switched the labels on the tubes and therefore the samples labeled as "control" might actually have been stained with the CD8 antibody. To check, I ran the experiment a second time, which verified the results of the first experiment and the high staining with the isotype control antibody.

It is unknown whether the company sent the wrong antibody, if something was wrong with their quality control, or if the wrong control antibody was listed on the company website. Either way, the lack of a reliable isotype control makes

it impossible to draw any conclusions about the percent of cells stained with the anti-CD8 antibody. In addition, the questionable behavior of one antibody brings into question the reliability of any other antibodies from the same company. Thus the percent of CD8$^+$ T-cells for BALB/c and C57BL/6 mice was not determined. Since this information was not essential to my primary question regarding expression of the IL-2 receptor, this aspect of the study was pursued no farther.

High Affinity IL-2R Expression on CD8$^+$ T-cells

Initially I wanted to look at expression of the IL-2 receptor on CD8$^+$ T-cells at four different time points; naïve, 1-week, 2-weeks, and 4-weeks infected. I used an antibody to CD25, which is the alpha subunit of the IL-2 receptor (IL-2Rα), to determine the presence the high affinity IL-2 receptor. In order to establish if any of these times were worth pursuing more than others and to see if any of the time points were acting similarly I started experiments using only one mouse of each strain per time of infection. Based on this preliminary data it appears that BALB/c and C57BL/6 mice have different expression patterns of IL-2Rα, and thus the high affinity IL-2 receptor.

The naïve mouse of each strain showed no expression of IL-2Rα, which was expected. The animals were not infected with the virus and they were kept in separate cages in the Mount Holyoke College Animal room, thus they should be healthy animals and not going through an immune response. Since the high

affinity IL-2 receptor is associated with activated T-cells, naïve, healthy animals should show no IL-2Rα expression.

At one week after infection, a difference was seen between the BALB/c and C57BL/6 mice. CD8^{+} T-cells from the BALB/c mouse were expressing IL-2Rα at a percentage much higher than the CD8^{+} T-cells from the C57BL/6 mouse, for which expression was almost negligible. This suggests activation of the CD8^{+} T-cells in the BALB/c mouse in response to the MuLV infection, but not in the C57BL/6 mouse.

In contrast to the mouse that had been infected for one week, the 2-week infected C57BL/6 mouse expressed comparatively high levels of IL-2Rα. The percent of CD8^{+} T-cells expressing IL-2Rα for the 2-week infected C57BL/6 mouse was more than triple the amount for the one-week infected BALB/c mouse; 26.1 percent for the 2-week infected C57BL/6 mouse as compared to 7.5 percent for the one-week infected BALB/c mouse. Unfortunately, CD8^{+} T-cells were not able to be isolated from the 2-week infected BALB/c mouse, so comparisons could not be made between the animals at 2-weeks, nor could it be observed whether changes occur between one-week infected and 2-week infected BALB/c mice. However, the 4-week infected BALB/c mouse shows no IL-2Rα expression on isolated CD8^{+} T-cells; therefore it appears that by 4 weeks after infection IL-2Rα expression has decreased back to naïve levels for CD8^{+} T-cells. The 4-week infected C57BL/6 mouse on the other hand still showed expression of IL-2Rα on CD8^{+} T-cells. Though the percent of cells expressing IL-2Rα was

lower than for the 2-week infected mouse, 19.5 percent compared to 26.1 percent, it was still more than twice the highest percent of IL-2Rα expression seen on CD8$^+$ T-cells for a BALB/c mouse.

These results correspond with what is known about disease progression in both strains. For the BALB/c mice there was a increase in IL-2Rα on CD8$^+$ T-cells one week following infection, which corresponds to an activated immune response that allows the animal to fight the virus. By 4 weeks after infection, when the animal is almost fully recovered, IL-2Rα expression is no longer seen on the CD8$^+$ T-cells, corresponding to the return to a healthy naïve state. The C57BL/6 mice show a different trend. There appears to be a delay in the activation potential of CD8$^+$ T-cells for these mice. While the BALB/c CD8$^+$ T-cells were expressing IL-2Rα by one week after infection, no significant IL-2Rα expression was seen for C57BL/6 mice until 2 weeks after infection. At that time, a dramatic increase in IL-2Rα expression on CD8$^+$ T-cells was observed and similar levels of expression persisted 4 weeks after infection. This agrees with the undirected and uncontrolled characteristics of a C57BL/6 mouse's immune response to MuLV and the lymphoproliferation that is one of the symptoms of MAIDS. When activation associated signals are triggered, they are perhaps triggered more than they need to be and if they are down-regulated it is not to a great enough extent.

In a study done by Makino *et al*. (1992), normally resistant A/J mice were depleted of CD8$^+$ T-cells using anti-CD8 antibodies, which resulted in

development of MAIDS. Antibody treatments were then stopped to see if

MAIDS would persist without continued suppression of the CD8$^+$ cells. Five

weeks after antibody treatments stopped, mice were found to have

lymphoproliferation and histopaholgic changes indicative of MAIDs (Makino *et*

al., 1992). Thus, perhaps the delay of only one week before CD8$^+$ T-cells of

C57BL/6 mice are activated is enough to set the path to immunodeficiency. In

addition, this suggested trend of behavior for IL-2Rα expression on CD8$^+$ T-cells

for the two strains supports one of the general hypotheses of the Stranford lab that

it is early differences in the immune responses of BALB/c and C57BL/6 mice

which play an important role in determining between recovery from MuLV

infection or progression to MAIDS.

CD25 Expression on 2-week Infected Spleen Leukocytes

After looking at the results of IL-2Rα on CD8$^+$ T-cells from just one

animal of each strain I decided to focus on the 2-week time point and repeat the

experiment using biological replicates for each strain. I decided to look more

closely at 2-week infected animals because of the high level of IL-2Rα expression

on the initial 2-week infected C57BL/6 mouse.

Unfortunately, I was not able to run the experiments as I planned. The

reagents necessary to run the samples in the Bioanalyzer had to be ordered and the

mice had already been infected for 2 weeks before the reagents could arrive.

Therefore, the mice were sacrificed at 2-weeks post-infection as planned and the

spleen leukocytes were frozen until the experiments could be run. However, low numbers of cells collected from the BALB/c mice, coupled with the amount of cells that are lost in the process of freezing and thawing, made it impractical to attempt to isolate CD8$^+$ T-cells from those samples.

Instead I tried to determine the expression of IL-2Rα on CD8$^+$ T-cells by using antibodies to both IL-2Rα and CD8. The hope was that this would eliminate the need to isolate the CD8$^+$ T-cells and also provide some data regarding IL-2Rα expression on non-CD8$^+$ lymphocytes. While the Bioanalyzer at Mount Holyoke can detect both red and green fluorescence, I was unable to find a green fluorescent antibody conjugate that fluoresces bright enough for the Bioanalyzer to detect it. Therefore I tried taking my stained cells to Amherst College to run them in the flow cytometer in the Williamson lab. I tried running samples of both thawed cells and freshly isolated cells. In both cases, only the CD8 antibody was detected. Since Bioanalzyer results indicate that the APC was not fluorescing very brightly, it is possible that the APC fluorescence was lost in the background fluorescence picked up by the flow cytometer. Since the flow cytometer would not work with my IL-2Rα antibody, and I was unable to isolate the CD8$^+$ T-cells, I decided to look at IL-2Rα (CD25) expression on all the spleen leukocytes that had been collected from the 2-week infected mice.

My results show a statistical significance ($p < 0.01$) between the percentage of BALB/c spleen leukocytes expressing CD25 and the percentage of C57BL/6 spleen leukocytes expressing CD25. The C57BL/6 mice have a higher

level of CD25 expression than do the BALB/c mice. CD25 was expressed on

35.4 (SD ± 14.6) percent of the C57BL/6 spleen leukocytes and 11.9 (SD ± 5.8)

percent of the BALB/c spleen leukocytes. Yet, this does not indicate whether

C57BL/6 mice have higher expression of the high affinity IL-2 receptor. This is

because there is a subset of T-cells known as T-regulatory cells (T_{regs}), which are

$CD4^+ CD25^+$. Thus, some of the cells expressing CD25 may be T_{regs} instead of

activated T-cells.

T-cells that are $CD4^+ CD25^+$ comprise a naturally occurring lymphocyte

population that suppresses the responses of other T-cells (Damoiseaux, 2006;

Nelson, 2004). Knowledge of this class of cells is relatively new and thus there is

still a lot that is not understood about the mechanism by which they regulate T-

cells or the role that IL-2 may play in their function. However, the fact that

CD25, a subunit of the IL-2 receptor, is a marker of this class of cells indicates

that IL-2 has some function relating to T_{reg} activity (Nelson, 2004). T_{regs} in the

periphery express all three of the IL-2 receptor subunits, so IL-2 might act as a

growth factor for peripheral T_{regs} (Nelson, 2004). Yet, other studies show that IL-

2 signaling is not required for T_{reg} function, and in fact it may even disrupt it

(Nelson, 2004). Of particular interest to this study, is the theory that HIV may

induce T_{regs} to inhibit virus-specific immune response (Damoiseaux, 2006).

So the question remains what specific populations of cells are being

observed by the CD25 expression on spleen leukocytes from BALB/c and

C57BL/6 mice. The higher percentage of cells expressing CD25 in C57BL/6 mice

could represent higher numbers of T_{regs}, which could be inhibiting the immune response making it a factor for susceptibility. On the other hand, the CD25 expression may be expression of the high affinity IL-2 receptor on either $CD4^+$ T-cells or $CD8^+$ T-cells. In addition, it may represent one population of cells in the BALB/c mice and a different population in the C57BL/6 mice. Thus, further work would be needed to classify the cells that are expressing CD25 in these animals and the roles that they play in the immune response.

Future Work and Conclusions

IL-2R Expression on $CD8^+$ T-cells

Despite many attempts, this study was unable to successfully describe the expression of the high affinity IL-2 receptor on $CD8^+$ T-cells of BALB/c and C57BL/6 mice. Very preliminary results from one animal of each strain suggest that there might be a different pattern of expression for the two strains of mice. In addition, the fact that there are no data for the 2-week infected BALB/c mouse makes it difficult to understand the full picture of how expression differs from 1 week to 4 weeks after infection. Given the time, I would have liked to gather data for that missing time point, as well as repeat the experiments on replicate animals to verify whether the trend that appears to be there truly does exist.

Cytokine Assays

Cells from each time point were also cultured and the supernatant was frozen for cytokine assays. Assays for IL-2 would provide a more complete

picture regarding the potential for T-cell activation during the immune response to MuLV. Comparisons could then be made between the amount of IL-2 present and the expression of the high affinity receptor at each time point to see what relationship exists for each animal. Lack of a strong proliferative response or inhibited stimulation of effector functions could result from, among other things, deficiency in IL-2 or limited expression of the high affinity IL-2 receptor. In addition, if IL-2 could serve to inhibit T_{regs} then IL-2 expression may have different implications depending on whether the higher percent of cells expressing CD25 in C57BL/6 represent activated T-cells or T_{regs}. Understanding these factors may help to see the larger picture relating to inefficient $CD8^+$ T-cell response during MuLV and HIV infections.

Broader Views

In more general terms, if the trend that appears to exist regarding different expression patterns of IL-2Rα on $CD8^+$ T-cells between BALB/c and C57BL/6 is real, it raises two sets of broad experimental questions. The first question is what are the upstream triggers that slow the response of C57BL/6 mice and/or allow BALB/c mice to have a quicker and more controlled response? The second question is what are the downstream effects of the delayed response in the C57BL/6 mice and how might they factor into the development of MAIDS? Additionally, are there other differences in these $CD8^+$ T-cell populations that allow for proper or impaired functioning?

It is generally accepted that CD8$^+$ T-cells play an important role in the immune system's fight against HIV and AIDS. The more we can learn about the way these cells are affected by HIV infection, and what impaired aspects of their functioning are associated with disease, the better chance we have at developing methods to counteract those deficiencies. Model systems such as MAIDS provide us with a place to start our search for the triggers and pathways key to disease pathogenesis. From there we can translate that knowledge to human applications and greater understanding of HIV and AIDS.

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APPENDIX

<u>Calculating Standard Deviation and Statistical Significance</u>

Percent of cells expressing CD25 = % gated for sample – % gated for control

Average (M) = $\dfrac{\sum \text{(Percent of cells expressing CD25)}}{\text{Number of samples}}$

$M_{BALB/c} = \dfrac{9.7 + 19.5 + 16.4 + 8.8 + 5.3}{5} = 11.9$

$M_{C57BL/6} = \dfrac{22.9 + 34.1 + 24.2 + 59.3 + 36.6}{5} = 35.4$

Standard Deviation (SD) = $\dfrac{\sum \text{(Percent of cells expressing CD25} - M)^2}{\text{Number of samples} - 1}$

$SD_{BABL/c} = 5.8$

$SD_{C57BL/6} = 14.6$

t-test

$M_{BALB/c} = \dfrac{.097 + .195 + .164 + .088 + .053}{5} = 0.119$

$M_{C57BL/6} = \dfrac{.229 + .341 + .242 + .593 + .366}{5} = 0.354$

$S^2{}_{BALB/c} = 0.003$

$S^2{}_{C57BL/6} = 0.021$

$S^2{}_{pooled} = (S^2{}_{BALB/c} + S^2{}_{C57BL/6}) / 2 = 0.012$

$S^2{}_{M} = 0.012 / 5 = 0.0024$

$S^2{}_{diff} = S^2{}_{M} + S^2{}_{M} = 0.0048$

$S_{diff} = \sqrt{S^2{}_{diff}} = 0.07$

$\mathbf{t} = (M_{C57BL/6} - M_{BALB/c}) / S_{diff} = \mathbf{3.36} \rightarrow \mathbf{p < 0.01}$