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## Analysis of CD8<sup>+</sup> T cell mediated anti-viral responses in mice with targeted deletions of the M<sub>1</sub> or M<sub>5</sub> muscarinic cholinergic receptors

Vaiva Vezys<sup>1</sup>, David Masopust<sup>1</sup>, Maxime Desmarests<sup>2</sup>, Jürgen Wess<sup>3</sup>, and James C. Zimring<sup>2,\*</sup>

<sup>1</sup> Emory Vaccine Center and the Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322

<sup>2</sup> Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

<sup>3</sup> Laboratory of Bioorganic Chemistry, NIH-NIDDK, DHHS, 8 Center Drive MSC 0810, Bethesda, Maryland, MD 20892-0810

### Abstract

A number of studies have demonstrated that non-neuronal acetylcholine can play a role in the regulation of T cell function. Recently, we reported that CD8<sup>+</sup> T cells, from mice with a targeted deletion of the M<sub>1</sub> muscarinic receptor, had a defect in differentiating into cytolytic T lymphocytes when stimulated *in vitro*. In the current report, we analyze the *in vivo* function of CD8<sup>+</sup> T cells from mice with targeted deletions of either M<sub>1</sub> or M<sub>5</sub> muscarinic receptors. M<sub>1</sub> or M<sub>5</sub> knockout mice were infected with either lymphocytic choriomeningitis virus or vesicular stomatitis virus. Expansion of anti-viral CD8<sup>+</sup> T cells was monitored by staining with tetramer reagents specific for the immunodominant peptides of the viruses. No defect in expansion of CD8<sup>+</sup> T cells was observed in either M<sub>1</sub> or M<sub>5</sub> knockout mice. The extent to which one can draw a generalized conclusion that M<sub>1</sub> and M<sub>5</sub> are not involved in anti-viral immunity depends upon issues of antigen strength, genetic background, induction of redundant receptors, and the potential for qualitative defects in the expanded CD8<sup>+</sup> T cells.

### Introduction

Over the past several decades, considerable evidence has accumulated to suggest that the function of T lymphocytes can be regulated by acetylcholine (ACh) (Kawashima and Fujii, 2003b; Kawashima and Fujii, 2004). It is possible that such regulation represents mechanisms by which the nervous system can influence immune function. However, ACh of neuronal origin is not a requirement, as it is becoming increasingly appreciated that ACh can be synthesized and secreted from non-neuronal sources (Kawashima and Fujii, 2003b; Kawashima and Fujii, 2004). Indeed, T lymphocytes have all the required machinery to synthesize, secrete, and respond to ACh, including choline acetyltransferase (Fujii et al., 1996; Fujii et al., 1998; Rinner et al., 1998), high affinity choline transporter (Fujii et al., 2003a), and both muscarinic and nicotinic receptors (Kawashima and Fujii, 2000; Kawashima and Fujii, 2003a). In addition, T cells express acetylcholinesterase (Szelenyi et al., 1982; Tayebati et al., 2002). Thus, all the

\* To whom all correspondence should be addressed: Please address correspondence to: James C. Zimring, M.D., Ph.D., Department of Pathology, Emory University, School of Medicine, Woodruff Memorial Research Building, Room 7301, 101 Woodruff Circle, Atlanta, GA 30322, USA (Telephone 404-712-2174, Fax 404-727-5764) Email jzimrin@emory.edu

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necessary components of a fully functional non-neuronal cholinergic system are found in lymphocytes.

An in-depth analysis of T cell lines derived from humans has demonstrated that muscarinic cholinergic receptors on T cells actively signal when stimulated with agonist (Kawashima and Fujii, 2000;Kawashima and Fujii, 2003a). Moreover, activation of muscarinic receptors has a functional outcome, as T cell receptor signaling in the presence of muscarinic agonists results in increased calcium flux, c-fos expression, IL-2 secretion, IL-2R expression, and proliferation (Kawashima and Fujii, 2000;Kawashima and Fujii, 2003a;Nomura et al., 2003;Rinner et al., 1995).

The five known muscarinic cholinergic receptors ( $M_1$ – $M_5$ ) can be subdivided into two general groups based upon signaling pathways.  $M_1$ ,  $M_3$  and  $M_5$  each signal through  $G_q$  proteins leading to activation of phospholipase  $C\beta$ , followed by generation of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Felder, 1995). In contrast, both  $M_2$  and  $M_4$  signal through  $G_i$  proteins leading to inhibition of adenylyl cyclase with a subsequent decrease in cyclic AMP (Felder, 1995). It has been demonstrated that these signaling pathways are intact in response to cholinergic stimulation of murine  $CD8^+$  T lymphocytes (Genaro et al., 1993).

The above observations were a major step forward in our understanding of regulation of T cells by ACh. However, they are mostly restricted to analysis of long-term immortalized cell lines. To extend these studies to the function of primary T cells, we have utilized mice with targeted deletions of each of the known muscarinic receptors ( $M_1$ – $M_5$ ). We have previously reported that  $CD8^+$  T cells from the  $M_1$  knockout mouse had a defect in the differentiation into cytolytic T lymphocytes (Zimring et al., 2005). No defect was observed in mice with targeted deletions of either  $M_3$ ,  $M_5$ , or a double deletion of  $M_2/M_4$  (Zimring et al., 2005).

Although our previous study demonstrated a functional defect in primary  $CD8^+$  T cells, it relied upon *in vitro* methodologies, carrying the advantage of an easily controlled reductionist system, but lacking the physiological relevancy of *in vivo* studies. In the current report, to extend our investigations into the *in vivo* setting, we infected muscarinic knockout mice with two different viruses, lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV), and examined *in vivo* expansion of anti-viral  $CD8^+$  T cells.

## Materials and Methods

### Mice

Mice with targeted deletions of the  $M_1$  or  $M_5$  receptors (Fisahn et al., 2002;Yamada et al., 2001) were backcrossed onto C57BL/6 mice at Taconic labs, for at least 10 generations. Wild-type C57BL/6 mice were purchased from Taconic labs. Mice were maintained on standard laboratory chow and water *ad libitum* in a temperature- and light-controlled environment. All procedures were approved by the Emory IACUC committee.

### Infection of Mice and Preparation of Cells

Injection of  $2 \times 10^5$  pfu LCMV strain Armstrong (i.p. injection) or  $1 \times 10^5$  pfu vesicular stomatitis virus (VSV) strain Indiana (i.v. injection) were given to mice of at least 8 weeks of age. As the peak of  $CD8^+$  T cell response to infection with LCMV and VSV occurs at approximately 7–10 days post-infection using the above inoculum, animals were sacrificed and analyzed at day 9 post infection. Splens were harvested and a single cell suspension was prepared after RBC lysis. Lymphocytes were counted using Trypan blue to exclude dead cells.

## Analysis of anti-viral CD8<sup>+</sup> T cells by flow cytometry

$1 \times 10^6$  splenocytes were stained with both anti-CD8 and MHC class I tetramers, consisting of the immunodominant epitopes of LCMV or VSV for the H-2<sup>b</sup> haplotype. Cells were incubated with tetramers specific for one of two different LCMV peptides presented by D<sup>b</sup> (D<sup>b</sup>-gp33–41; D<sup>b</sup>-gp276–286), or tetramer specific for the immunodominant VSV peptide (K<sup>b</sup>-VSV–N 52–59) for 30 min at 4°C. Antibodies against CD8  $\alpha$  (BD Pharmingen, San Diego, CA) were used according to manufacturer's directions. All data was acquired on a FACSCalibur (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (Ashland, OR).

## Results

### Analysis of CD8<sup>+</sup> T cell responses to LCMV and VSV in M<sub>1</sub> or M<sub>5</sub> knockout mice

Our previous findings demonstrated that naïve CD8<sup>+</sup> T cells from C57BL/6 mice express mRNA for both the M<sub>1</sub> and M<sub>5</sub> muscarinic receptors, whereas there was no detectable mRNA for M<sub>3</sub> (Zimring et al., 2005). Accordingly, we focused our studies on mice with targeted deletions of either M<sub>1</sub> or M<sub>5</sub>, on a C57BL/6 background. To test the role of M<sub>1</sub> or M<sub>5</sub> receptors in the ability of CD8<sup>+</sup> T cells to expand in response to a viral infection, M<sub>1</sub> or M<sub>5</sub> knockout mice were infected with either LCMV or VSV. At 9 days post-infection, leukocytes were isolated from the spleens of infected animals. CD8<sup>+</sup> T cells were visualized by staining cells with anti-CD8 and analyzing by flow cytometry. Within the CD8<sup>+</sup> population, T cells specific for LCMV or VSV were visualized by staining with tetramer reagents consisting of peptide/MHC I complexes known to represent the immunodominant antigens for LCMV or VSV, respectively.

Robust expansion of anti-viral CD8<sup>+</sup> T cells, specific for LCMV or VSV, was observed in all infected animals. This was visualized as a CD8<sup>+</sup>, tetramer<sup>+</sup> population (figure 1). This population was specifically induced by viral infection, as no tetramer positive population was observed in uninfected control mice (data not shown). The flow cytometry plots shown in figure 1 are representative of the typical results. Combined results of the absolute numbers of anti-viral CD8<sup>+</sup> T cells from multiple animals demonstrated no significant difference between M<sub>1</sub> knockout, M<sub>5</sub> knockout, or wild-type mice (Figure 2). Thus, neither M<sub>1</sub> nor M<sub>5</sub> receptors are required for the expansion of antigen-specific CD8<sup>+</sup> T cells in response to viral infection.

## Discussion

The data presented in this report indicate that CD8<sup>+</sup> T cells undergo normal expansion in mice with targeted deletions of either the M<sub>1</sub> or M<sub>5</sub> cholinergic receptors. Although these data assess the quantity of anti-viral CD8<sup>+</sup> T cells that expand in infected mice, the current approach does not assess effector function of the anti-viral cells. Ongoing studies to assess cytolytic T lymphocyte (CTL) activity and viral loads will help to address this issue.

We have previously reported that mRNA for both M<sub>1</sub> and M<sub>5</sub> are expressed in naïve CD8<sup>+</sup> T cells from C57BL/6 mice (Zimring et al., 2005). Along these lines, one must also consider the possibility of redundancy between M<sub>1</sub> and M<sub>5</sub> such that they compensate for the absence of each other. We have previously reported a defect in CD8<sup>+</sup> T cells from mice lacking the M<sub>1</sub> receptor (Zimring et al., 2005), indicating that M<sub>5</sub> did not rescue the defect in M<sub>1</sub> knockout mice. However, it is important to note that these studies were performed *in vitro* and on a different strain background from the current studies. It is possible that M<sub>5</sub> can compensate for the absence of M<sub>1</sub> *in vivo* and/or on a C57BL/6 background. Thus, a double M<sub>1</sub>/M<sub>5</sub> knockout may uncover a functional defect in CD8<sup>+</sup> T cells. Moreover, although no mRNA for M<sub>3</sub> was detected in naïve CD8<sup>+</sup> T cells (Zimring et al., 2005), as muscarinic receptor expression can

change upon T cell activation (Fujii et al., 2003b), it is also possible that CD8<sup>+</sup> T cells express M3 upon activation. To definitively address this issue, a triple knockout of M<sub>1</sub>, M<sub>3</sub> and M<sub>5</sub> will be required. As such an animal would not likely be viable, conditional knockouts in the CD8<sup>+</sup> T cell compartment would be most useful for these purposes.

The studies in this report were carried out using LCMV and VSV, as they are well characterized pathogens with sufficient immunological tools to perform *in vivo* analysis of CD8<sup>+</sup> T cells. However, it is important to note that our previous analysis indicated that the defect in CD8<sup>+</sup> T cells from mice missing M<sub>1</sub> was not absolute (Zimring et al., 2005). CD8<sup>+</sup> T cells from M<sub>1</sub> knockout mice showed a clear defect when given a weak to moderate *in vitro* stimulation. However, a strong stimulation of the TCR could overcome the defect (Zimring et al., 2005), resulting in normal CTL function. As both LCMV and VSV represent a strong stimulus to the immune system, it is possible that like our previous *in vitro* studies, defects in muscarinic receptor knockout mice were overcome due to the strength of the stimulus. Ongoing studies are addressing this hypothesis by challenging mice with weaker stimuli, such as immunization to minor histocompatibility antigens.

As is often the case with characterizations of knockout mice, there is some concern regarding the genetic background of the M<sub>1</sub> and M<sub>5</sub> knockout mice. Our previous report that demonstrated a defect in CD8<sup>+</sup> T cells from M<sub>1</sub> knockout mice utilized animals on an outbred background (Zimring et al., 2005). As many well-characterized tetramer reagents containing immunodominant epitopes have been described for the H-2<sup>b</sup> haplotype, for the current studies, it was necessary to have the knockout mice on a genetic background that was H-2<sup>b</sup>. Accordingly, the current studies utilized M<sub>1</sub> and M<sub>5</sub> knockout mice backcrossed onto a C57BL/6 background. It has been reported that the pattern of expression of muscarinic receptor subtypes on human leukocytes varies from individual to individual (Kawashima and Fujii, 2003b; Sato et al., 1999). Thus, it seems likely that patterns of muscarinic receptor expression varies between different inbred murine strains. As the M<sub>1</sub>, M<sub>3</sub> and M<sub>5</sub> receptors each signal by redundant pathways, knockout of a single receptor may result in a phenotype in some but not other strains of mice. This possibility is further complicated by the observations that expression of muscarinic receptors is altered during T cell activation (Fujii et al., 2003b), which may also differ between strains.

In summary, the data presented herein demonstrate that neither M<sub>1</sub> nor M<sub>5</sub> are required for expansion of antigen-specific CD8<sup>+</sup> T cells, in response to viral infection in C57BL/6 mice. As explained above, the extent to which one can draw a generalized conclusion that M<sub>1</sub> and M<sub>5</sub> are not involved in anti-viral immunity mediated by CD8<sup>+</sup> T cells, depends upon issues of antigen strength, genetic background, and receptor redundancy.

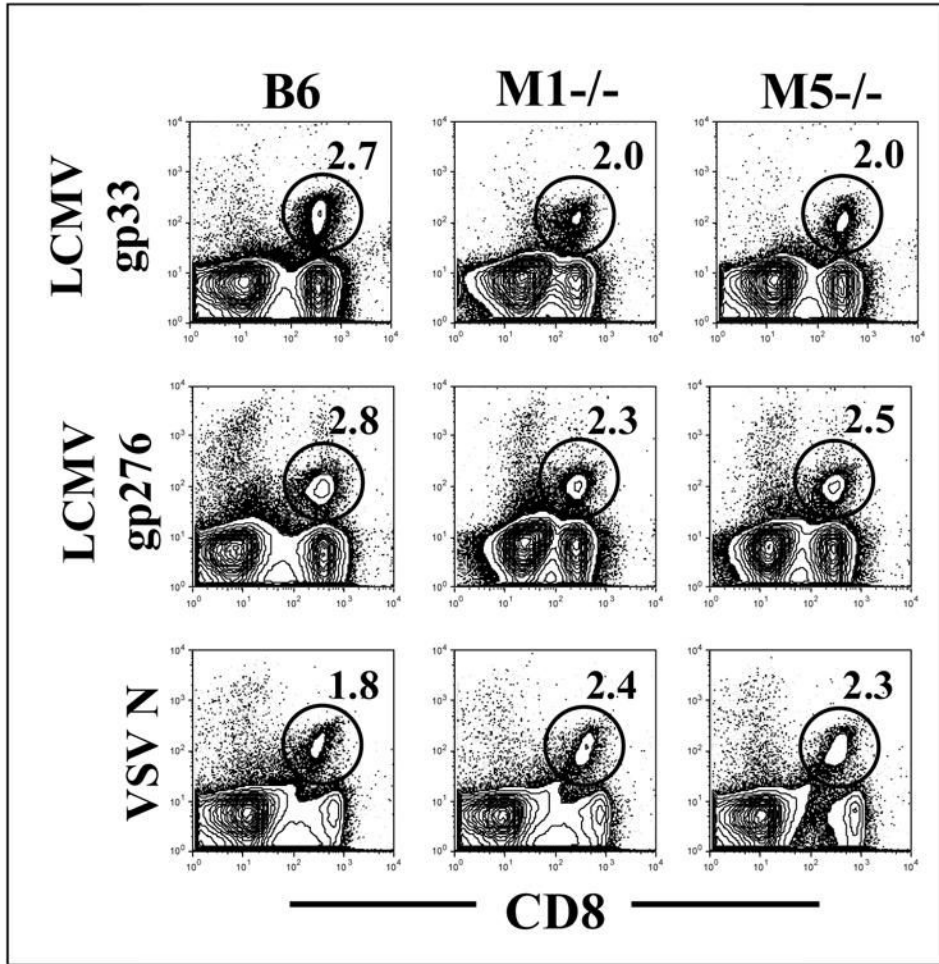
#### Acknowledgements

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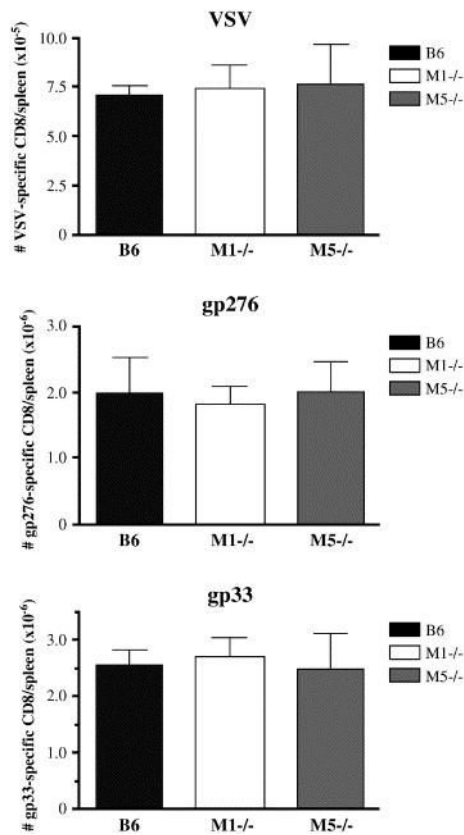
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**Figure 1. Visualization of anti-viral CD8<sup>+</sup> T cells in mice with deletions of the M1 or M5 muscarinic receptors**

Mice were infected either with LCMV or VSV. Nine days later, splenocytes were stained with anti-CD8 antibody and tetramer specific for LCMV (gp33 or gp276) or VSV. Flow plots are gated on total lymphocytes and CD8<sup>+</sup> tetramer<sup>+</sup> cells were visualized (shown in circles) The number within the plots reflects the percent of lymphocytes which bind the indicated tetramer.



**Figure 2. Expansion of anti-viral CD8<sup>+</sup>T cells in mice with deletions of the M1 or M5 muscarinic receptors**

The absolute numbers of CD8<sup>+</sup> tetramer<sup>+</sup> cells were averaged from multiple animals. Numbers in bar graphs are calculated by multiplying the total number of splenocytes by the percent of lymphocytes binding a particular tetramer. For the experiment shown, sample size was n=3 for B6 infected with LCMV or VSV, n=3 for M1<sup>-/-</sup> infected with LCMV, n=5 for M5<sup>-/-</sup> infected with LCMV, n=4 M1<sup>-/-</sup> infected with VSV and n=4 M5<sup>-/-</sup> infected with VSV. This experiment has been reproduced with similar results.