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$CD4^+$ cytolytic effectors are inefficient in the clearance of *Listeria monocytogenes*

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SUMMARY

Cytotoxic T lymphocytes (CTL) recognize and lyse target cells through the interaction of the T-cell receptor complex with the class I or class II major histocompatibility complex (MHC). The production of class I-restricted CTL has been shown to be critical to the elimination of specific pathogens including *Listeria monocytogenes*. However, the function of class II-restricted CTL in the clearance of intracellular pathogens is poorly understood. H-2^b β_2-microglobulin-deficient mice (β_2M−/−) are not able to produce $CD8^+$ CTL in response to infection with *L. monocytogenes*. We used this model to evaluate the efficacy of class II-restricted CTL, in the absence of a class I-restricted response, during a primary infection with *L. monocytogenes*. We demonstrate that, despite their effectiveness in adoptive transfer of protection, *Listeria*-specific $CD4^+$ class II-restricted cytotoxic lymphocytes are ineffective in decreasing titres of *L. monocytogenes* in the spleen after an established infection. In β_2M−/− mice, persistence of *L. monocytogenes* in the spleen was found preferentially in class II-negative cells. Surprisingly, class I-restricted CTL from C57BL/6 mice were capable of decreasing bacterial titres during an established infection even in the absence of detectable class I on the surface of cells from β_2M−/− mice. These data strongly suggest that, in the absence of a class I-restricted response, pathogens that elicit a class II-restricted cytotoxic response may escape prompt eradication by the immune system.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) recognize class I and class II major histocompatibility complexes (MHC) on target cells via the T-cell receptor (TCR)–CD3 complex.[1] Interaction of the TCR on the CTL with the MHC molecule on the target cell in the presence of a costimulatory signal, can lead to death of the target cell.[2] Both class I and class II-restricted CTL are capable of directly mediating lysis of target cells. $CD8^+$ class I-restricted CTL have been shown to be important in the lysis of target cells infected with virus and in the surveillance of tumour cells.[3–5] In contrast, the expansion of class II-restricted CTL, in the absence of a class I-restricted response, is observed during infection with herpes simplex virus.[6,7] This infection is characterized by persistence of infection because of incomplete clearance of the virus.

The selection of class I-restricted $CD8^+$ T cells from double-positive T cells in the thymus is dependent on the presence of class I complexes on presenting cells.[8] Two different laboratories have produced mice that have an inactivated gene for β_2-microglobulin (β_2M).[9,10] Because β_2M is necessary for the stability of the class I MHC complex during thymic selection, β_2M−/− mice have a profound decrease in the number of $CD8^+$ T cells with normal numbers of $CD4^+$ and $\gamma\delta$ T cells.[11]

Previous work in our laboratory and in those of others had demonstrated that β_2M−/− mice produce vigorous $CD4^+$ T-cell responses after viral infection.[12,13] Kaufmann *et al.* had previously demonstrated that H-2^b mice can produce *Listeria*-specific $CD4^+$ class II-restricted CTL.[14] The ability of these $CD4^+$ CTL to substitute for $CD8^+$ CTL in the clearance of a primary infection with an intracellular pathogen has not been assessed. Because H-2^b β_2M−/− mice do not produce $CD8^+$ CTL in response to infection with *L. monocytogenes*, we used this model to evaluate the role and function of class

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Abbreviations: β_2M−/−, β_2-microglobulin knock-out mouse; Con A supernatant, concavalin A-stimulated supernatant; CTL, cytotoxic T lymphocyte; LM, *Listeria monocytogenes*.

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II-restricted CTL during a primary infection in the absence of a class I-restricted response.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2K^{b}A^{b}E^{o}D^b) mice and B10.A(5R) (H-2K^{b}A^{b}E^{k}D^d) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All of the β_2M$-/-$ mice were utilized between generations F6 and F9, and at 7–12 weeks of age. All mice were housed under microisolator cage tops and maintained specific-pathogen-free. Mice were cared for at the animal care facility of the University of North Carolina at Chapel Hill which is an American Association for Laboratory Animal Care-approved facility.

Bacteria

L. monocytogenes, strain EGD, was prepared as previously described.[15] After infection, mice were sacrificed and the spleens and livers removed. One-quarter of each organ was processed for histology by fixation in neutral-buffered formalin. The remainder of each organ was homogenized in phosphate-buffered saline with 0·1% Triton-X-100. The homogenate was plated on trypticase soy agar (TSA) and the number of bacteria recovered was quantified. Virulent *L. monocytogenes* was maintained by passage through β_2M$-/-$ mice.

Histology

Tissue samples were formalin-fixed, paraffin-embedded, processed and stained following standard histologic procedures for light microscopic evaluation. *L. monocytogenes* was localized in formalin-fixed tissue sections using a streptavidin–biotin immunoperoxidase technique.[16]

Monoclonal antibodies

Anti-CD4 (GK1.5) (kindly provided by Dr Philip Cohen, UNC-Chapel Hill)[17] and anti-interferon-γ (IFN-γ) (R4-6A2)[18] (American Type Tissue Collection HB 170) monoclonal antibodies were purified from overgrown supernatants.[19] The presence of antibody was verified by Coomassie Blue staining of the preparation on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels.[20] Monoclonal antibodies 7.16.30 (IgM reactive with I-A^b), 7.16.17 (IgG reactive with I-A^b), 11.3.1 (IgM reactive with K^b) 4.2.3 (IgM reactive with K^f)[21] and 28-8-6s (IgG reactive with H-2K^b,D^b; ATCC HB51)[22] were used as overgrown supernatants.

Cell lines

IC-21 (H-2^b), J774.1 (H-2^d), YAC-1 and Ltk- cell lines were obtained from American Type Culture Collection (Rockville, MD). B10.A(5R) macrophages were grown from bone marrow as previously described.[23] Cell lines were maintained in RPMI (Gibco, Grand Island, NY) supplemented with heat-inactivated 10% fetal calf serum (v/v) (Gibco, Grand Island, NY), 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol (ME), (R10). Concanavalin A (Con A)-stimulated supernatant was generated as previously described.[19] Con A-stimulated supernatant was titered for activity on the interleukin-2 (IL-2)-dependent cell line HT-2.[19]

CTL lines from β_2M$-/-$ mice were generated using a standard protocol. Seven or eight days after infection, spleens were removed from β_2M$-/-$ mice and resuspended in R10. Splenocytes (10^6) were incubated with 10^7 irradiated (3000 rads) syngeneic spleen cells that had been infected with live *L. monocytogenes* using a previously described method.[24] Six days after stimulation with antigen, 15% Con A supernatant was added to each well. The CTL lines were stimulated in this manner two or three times prior to use in ^{51}Cr release assays.

For the generation of CTL lines from C57BL/6 mice, IC-21 macrophages (feeders) were infected with *L. monocytogenes* and irradiated at 7000 rads prior to use. C57BL/6 mice were infected with *L. monocytogenes*, 7 days later the mice were killed and the splenocytes were depleted of $CD4^+$ cells using two rounds of monoclonal antibody GK1.5 plus rabbit complement (Cedar Lane, Hornby, Canada) followed by positive selection by panning on $CD8^+$ coated plates.[25] These splenocytes were incubated with irradiated *L. monocytogenes*-infected IC-21 feeders. Twenty-four hours later, 30% Con A supernatant was added to each well. The cell lines were restimulated at 7–10-day intervals as described with fresh *L. monocytogenes*-infected feeder cells and 15% Con A supernatant.

Flow cytometry

Flow cytometry was performed using a fluorescence-activated cell sorter scan (FACScan) (Becton-Dickinson, San Jose, CA). CD4 fluorescein isothiocyanate (FITC)-conjugated, CD8-FITC-conjugated, CD3ε–phycoerythrin (PE)-conjugated and Thy 1.2 biotin-conjugated were purchased from Pharmingen (San Diego, CA). Red 670 conjugated to strepavidin was used as the second step for the Thy 1.2 labelling (Gibco, Grand Island, NY). Histograms were analysed using Cicero software (Cytomation, Fort Collins, CO).

^{51}Chromium-release (Cr-release) assay

^{51}Cr-release assays were performed as previously described.[26] Target cells were incubated for 3 hr with live *L. monocytogenes* as indicated and subsequently, 10^6 cells incubated with 200 μCi of ^{51}Cr for 45 min. Five thousand target cells were added per well. ^{51}Cr-release assays were performed at least 96 hr after antigen-stimulation of the CTL.

Adoptive transfer

Lymphocytes were separated from feeder cells by centrifugation over lympholyte-M (Cedar Lane, Hornby, Canada). Ten million cultured T lymphocytes (referred to as CTL although all of the cells may not be cytotoxic) were transferred by intravenous (iv) injection into β_2M$-/-$ mice. Forty-five minutes later, 10× the LD_{50} of *L. monocytogenes* (5×10^4 organisms) were injected i.v. In addition, some β_2M$-/-$ mice were treated with 200 μg of anti-IFN-γ monoclonal antibody (R4-6A2) on days -1 and 1 hr prior to the injection of CTL. Mice were either followed until death or sacrificed at 96 hr and the number of bacteria in the spleen was quantified. Control mice received splenocytes from non-primed animals.

Cell phenotype responsible for harbouring persistent organisms

β_2M$-/-$ mice and C57BL/6 mice were infected with *L. monocytogenes* at 0·3 × LD_{50} respectively (3×10^3 organisms for C57BL/6) for the mice sacrificed at day 1 and 0·5 × LD_{50} for mice sacrificed at day 7. The spleens were homogenized in

RPMI 5% fetal calf serum without antibiotics and aliquoted into five fractions. Fractions were treated with two rounds of monoclonal antibodies 7.16.30 (IgM reactive with I-A^b) or 11.3.1 (IgM reactive with K^b) respectively followed by rabbit complement. Supernatants were collected and plated serially on trypticase soy agar (TSA) plates. Control fractions were treated with either phosphate-buffered saline (PBS) containing 0·1% Triton-X-100 or maintained in media alone. The final fraction was treated with an isotype similar irrelevant monoclonal antibody (4.2.5 IgM reactive with K^f). Adequacy of lysis was assessed by flow cytometry (data not shown).

Statistical analysis

P values for mice in the adoptive transfer experiments were analysed using Fisher's exact test with two-tail analysis. Log values in the spleen after adoptive transfer were assessed using Student's *t*-test.

RESULTS

Generation of *L. monocytogenes*-specific CTL lines

CTL lines were produced from both C57BL/6 mice and β_2M−/− mice. CTL from lines in C57BL/6 mice were MHC-restricted and antigen specific (Fig. 1). There was no activity with MHC-mismatched H-2^d target cells (J774.1), or MHC-matched non-infected targets. CTL lysed macrophages from B10.A(5R) mice (H-$2K^bA^bE^kD^d$). CTL lysis was blocked by the monoclonal antibody 28-8-6s (reactive with K^b, D^b) and not by the monoclonal antibody 7.16.17 (reactive with I-A^b) (Fig. 1). Lysis of infected IC-21 and 5R macrophages maps the CTL response to either H-$2K^b$ or A^b. The decreased lysis observed upon the addition of antibody 28-8-6s shows that CTL from C57BL/6 mice are restricted by the class I molecule K^b. Flow cytometry (data not shown) confirmed that the population was $CD8^+/CD3^+/CD4^-$.

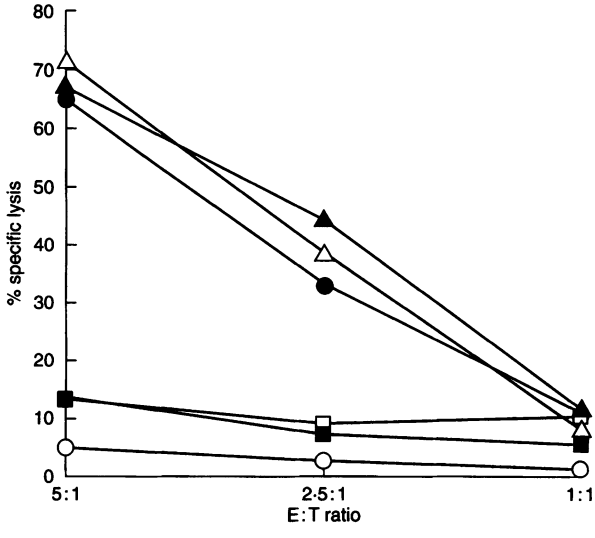

Figure 1. Specific lysis of target cells by CTL lines from C57BL/6 mice. CTL lines were generated as described. After two *in vitro* stimulations, CTL were tested in a standard ^{51}Cr-release assay. Target cells were IC-21 (H-2^b) infected with *L. monocytogenes* (filled triangle); IC-21 uninfected (open square); J774.1 (H-2^d) infected with *L. monocytogenes* (open circle); IC-21 infected with *L. monocytogenes* and treated with monoclonal antibodies 7.16.17 (IgG reactive with I-A^b) (open triangle) or 28-8-6s (IgG reactive with K^b, D^b) (solid square) and macrophages from B10.A(5R) mice infected with *L. monocytogenes* (filled circle). For each experiment, 5 μg of antibody was added 30 min prior to the addition of effector cells. Lysis of uninfected J774.1 and B10.A(5R) macrophages was less than 5% at an E:T ratio of 5:1 (not shown). Spontaneous release for each target cell was less than 30%. Representative data of one of five experiments is shown.

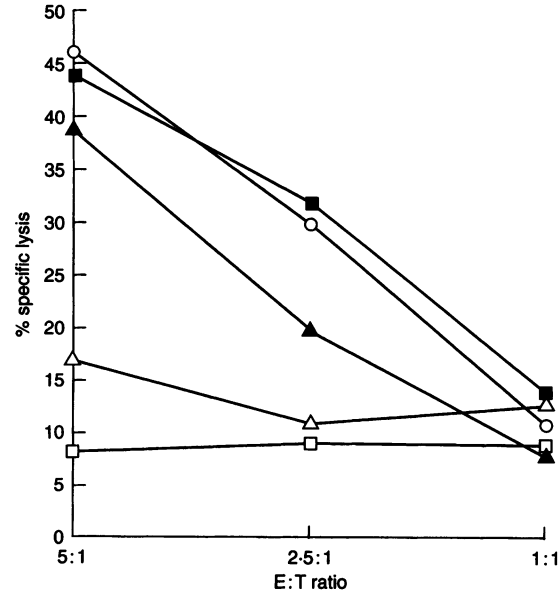

Figure 2. Specific lysis of target cells by CTL lines from the β_2M−/− mice. CTL's from β_2M−/− mice were tested for their ability to lyse target cells in a standard ^{51}Cr-release assay. Target cells were IC-21 (H-2^b) infected with *L. monocytogenes* (filled triangle); IC-21 uninfected with *L. monocytogenes* (open square); 5R macrophages (H-$2K^bA^bE^kD^d$) infected with *L. monocytogenes* (open circle); IC-21 infected with *L. monocytogenes* and treated with monoclonal antibodies 7.16.17 (open triangle) or 28-8-6s (filled square). For each experiment, 5 μg of antibody was added for 30 min prior to the addition of the effector cells. Control J774.1 cells (H-2^d) infected with *L. monocytogenes* or uninfected and infected 5R macrophages demonstrated less than 10% specific lysis at an E:T ratio of 5:1 (not shown). Spontaneous release for all targets was less than 25%. This is a representative data of one in three experiments.

CTL from β_2M−/− mice possessed a different pattern of killing. These CTL lysed the H-2^b cell line IC-21 after infection with live *L. monocytogenes* (Fig. 2). There was no appreciable lysis of either non-infected targets or the H-2^d macrophage line J774.1 (data not shown). This showed that the response is MHC-restricted and antigen specific. Unlike the CTL from C57BL/6 mice, we were unable to block the lysis of the IC-21 cell line by β_2M−/− effectors with monoclonal antibody 28-8-6s (reactive with K^b, D^b). In contrast, we were able to decrease the specific lysis of β_2M−/− effectors nearly to baseline using the monoclonal antibody 7.16.17 (reactive with I-A^b) (Fig. 2). The CTL from β_2M−/− mice lysed infected macrophages from 5R ($K^bA^bE^kD^d$) mice. These data indicate that the CTL generated in β_2M−/− mice were I-A^b-restricted. CTL from β_2M−/− mice did not lyse the NK-sensitive cell line YAC-1 (data not shown). Flow cytometry of CTL from β_2M−/− mice showed that 98% of the CTL were $CD3^+/4^+8^-$. Only 2% of

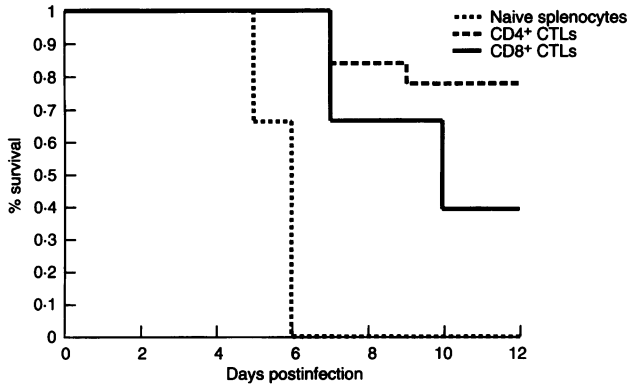

Figure 3. Survival of β_2M−/− mice infected with a lethal dose of *L. monocytogenes* prior to the adoptive transfer of $CD4^+$ or $CD8^+$ killer cells. β_2M−/− mice were infected i.v. with 10× the LD_{50} of the mouse for *L. monocytogenes* (5×10^4 organisms). Forty-five minutes later the animals were given 10^7 CTL from C57BL/6 ($CD8^+$) or β_2M−/− ($CD4^+$) mice that had been generated as described. Animals were followed for 14 days. Animals that were moribund were sacrificed and the data censored from that point. Data is pooled from three experiments; $n = 6$ mice per experiment.

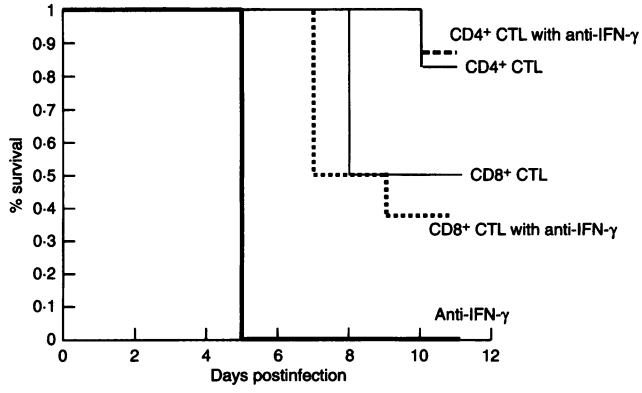

Figure 5. Survival of mice in the adoptive transfer experiments is not dependent on the generation of IFN-γ. Prior to the inoculation of *L. monocytogenes*, β_2M−/− mice were given 200 μl of α-IFN-γ ip on day −1 and one hour prior to infection. Following this, the animals were inoculated with *L. monocytogenes* iv and administered CTL lines i.v. in adoptive transfer experiments as described above. Animals in these experiments were given 3× the LD_{50} ($1{\cdot}5 \times 10^3$) for *L. monocytogenes*. In each group, $n = 8$ mice treated with the α-IFN-γ antibody and given either $CD4^+$ or $CD8^+$ effector cells. $N = 6$ mice treated only with *L. monocytogenes* and the $CD4^+$ or $CD8^+$ effector cells. The mice treated with antibody plus *L. monocytogenes* ($n = 4$). Antibodies were generated as described in the methods. Control mice which died by day 5 were given 0·5× the LD_{50} and 200 μl × 2 of the α-IFN-γ prior to infection.

the CTL expressed the γδ T-cell receptor. None of the CTL from the lines tested ($n = 5$) expressed the CD8 coreceptor (data not shown).

Adoptive transfer of $CD4^+$ and $CD8^+$ populations to lethally infected β_2M−/− mice

Mice were inoculated i.v. with 10 × LD_{50} for β_2M−/− mice and 45 min later given 1×10^7 CTL i.v. that were either $CD4^+$ (from β_2M−/− mice) or $CD8^+$ (from C57BL/6 mice) and followed for 14 days or until death. Control mice were treated with 1×10^8 splenocytes from naive β_2M−/− or C57BL/6 mice. All of the mice treated with control splenocytes died by day 5 after infection (Fig. 3).

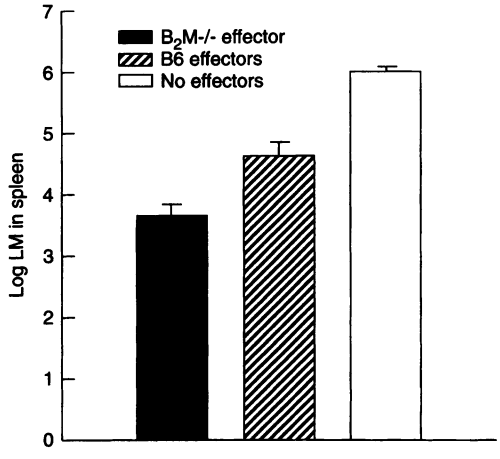

Figure 4. Administration of CTL lines from C57BL/6 and β_2M−/− mice at the time of a lethal infection decreases CFU in the spleen. β_2M−/− mice were infected with 10× the LD_{50} (5×10^4) for *L. monocytogenes* and 45 min later administered 10^7 CTL from C57BL/6 ($CD8^+$) or β_2M−/− ($CD4^+$) mice. Ninety-six hours later the spleens were removed homogenized and plated. Growth of *L. monocytogenes* was assessed at 18 and 24 hr by visual inspection. Control mice were given splenocytes from naive β_2M−/− or C57BL/6 mice. Data is representative of two experiments and is expressed as the mean of the log of CFU in the spleen with standard error bars. $N = 4$ mice per condition.

Survival was greatest for β_2M−/− mice given the $CD4^+$ class II-restricted population of CTL from β_2M−/− mice. Fourteen of 18 (pooled data from three separate experiments) mice survived the infection (Fig. 3). This result was statistically significant when compared to the control mice ($P < 0{\cdot}0001$). We found that mice that received $CD8^+$ *L. monocytogenes*-specific CTL from C57BL/6 mice had an increased survival over control mice with 7 of 18 mice surviving (Fig. 3). This result was statistically significant when compared to control mice ($P = 0{\cdot}005$). $CD4^+$ CTL were more effective than $CD8^+$ CTL in the adoptive transfer of protection ($P < 0{\cdot}05$). The administration of $CD8^+$ CTL from C57BL/6 mice was not protective in H-2 mismatched BALB/c ($H\text{-}2^d$) mice (data not shown).

To check for quantitative effects, we determined the number of organisms in the spleens of β_2M−/− mice given $CD4^+$ (from β_2M−/−) or $CD8^+$ (from C57BL/6) CTL after a lethal infection with *L. monocytogenes*. We confirmed that $CD4^+$ CTL could mediate the greatest protection via adoptive transfer (see Fig. 4) ($P < 0{\cdot}001$). $CD8^+$ killers were able to mediate a modest decrease in *L. monocytogenes* ($P = 0{\cdot}01$) as compared to the administration of naive splenocytes. There was a statistically significant difference between the effect of $CD4^+$ CTL and $CD8^+$ CTL ($P = 0{\cdot}01$).

Role of interferon-γ

IFN-γ is crucial to the *in vivo* murine response to *L. monocytogenes*. $CD4^+$ and $CD8^+$ lymphocytes are capable of producing IFN-γ.[27] We were interested in determining if the

production of IFN-γ was the principle mechanism for the adoptive transfer of protection by $CD8^+$ and $CD4^+$ killer cells. In preliminary experiments, we evaluated the effect of blocking IFN-γ activity on a sublethal infection of *L. monocytogenes*. We found that all of the mice ($n = 3$, data not shown) treated with 200 μl i.v. of the monoclonal antibody R4-6A2 on days -1 and 0 died by day 5 after a sublethal infection with *L. monocytogenes*. This antibody treatment had no effect on animals inoculated with saline. We used this dose and schedule of administration of R4-6A2 to investigate the role of IFN-γ in the adoptive transfer of protection by $CD4^+$ and $CD8^+$ CTL. We found no difference in protection via adoptive transfer using either $CD8^+$ or $CD4^+$ CTL (Fig. 5). All of the animals treated with the monoclonal antibody and a sublethal infection of *L. monocytogenes* died by day 5 confirming the efficacy of the antibody in neutralizing the activity of IFN-γ. Thus, neutralizing IFN-γ had no effect on the ability of either $CD4^+$ or $CD8^+$ cells to mediate protection in adoptive transfer.

Effect of $CD4^+$ and $CD8^+$ T cells on persistent infection

$\beta_2M-/-$ mice infected with *L. monocytogenes* are unable to resolve the infection rapidly. We determined if the transfer of additional *L. monocytogenes*-specific CTL at days 1 and 7 could result in an increased clearance of *L. monocytogenes* from the spleen of these mice. It was found that 1×10^7 $CD4^+$ CTL were able to mediate a modest reduction in *L. monocytogenes* titres when administered 18 hr after a sublethal infection ($P = 0{\cdot}05$) (Fig. 6). However, at 18 hr postinfection, 1×10^7 $CD8^+$ CTL were much more effective in decreasing titres of *L. monocytogenes* from the spleen as compared to $CD4^+$ CTL ($P < 0{\cdot}0001$) (Fig. 6). When given 7 days after a sublethal infection, 1×10^7 $CD4^+$ CTL from $\beta_2M-/-$ mice had no effect on the persistent

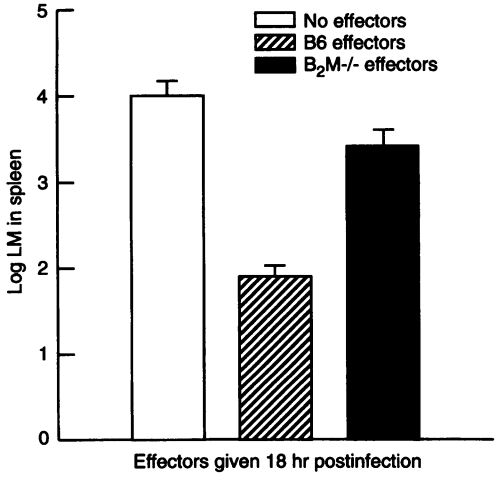

Figure 6. Effect of the administration of C57BL/6 CTL lines or $\beta_2M-/-$ lines 18 hr after a sublethal infection. $\beta_2M-/-$ mice were infected with $0{\cdot}3\times$ LD_{50} for *L. monocytogenes* ($1{\cdot}5 \times 10^3$). Eighteen hours postinfection the mice were given 10^7 CTL from either C57BL/6 ($CD8^+$) or $\beta_2M-/-$ mice ($CD4^+$). Sixty hours later the animals were sacrificed and the splenic homogenates plated. CFU were counted 18 hr after plating. All data is referenced to control animals administered naive C57BL/6 splenocytes; $n = 4$ animals per condition. Data is presented as the mean of the log of CFU in the spleen of the animals with standard error bars.

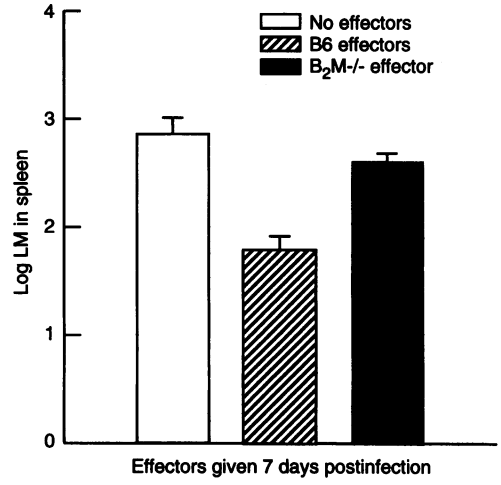

Figure 7. Effect of the administration of C57BL/6 and $\beta 2M-/-$ CTL lines 7 days after a sublethal infection with *L. monocytogenes*. These experiments were performed as those in Fig. 7. Mice were infected with $0{\cdot}5\times$ LD_{50} of *L. monocytogenes* and 7 days after the infection given CTL from either C57BL/6 or $\beta_2M-/-$ mice. Animals were sacrificed 60 hr after the administration of the CTL. Plates were visually read at 18 and 24 hr. $N = 5$ mice per condition.

titres of *L. monocytogenes* as compared to mice receiving naive splenocytes ($P > 0{\cdot}2$) (Fig. 7). By comparison, (Fig. 7) 1×10^7 $CD8^+$ CTL from C57BL/6 mice were able to affect a decrease in the number of *L. monocytogenes* in the spleen of $\beta_2M-/-$ mice when compared to naive splenocytes ($P = 0{\cdot}001$). Preliminary data (Serody *et al.*, unpublished) show that *L. monocytogenes*-specific $CD4^+$ class II-restricted CTL do not decrease the titres of *L. monocytogenes* in either the spleen or liver when administered 24 hr after infection.

Persistence of infection

To determine the cell surface phenotype of splenocytes harbouring *L. monocytogenes*, spleen cells from C57BL/6 and $\beta_2M-/-$ mice were treated with antibodies to $H\text{-}2K^b$ or A^b plus complement. At 18 hr postinfection, (Fig. 8a) infected splenocytes in C57BL/6 mice express both class I and class II. Because $\beta_2M-/-$ mice express extremely low levels of class I MHC molecules, the infected splenocytes only expressed $I\text{-}A^b$. After 7 days, the overall level of infection was reduced in C57BL/6 mice as compared to $\beta_2M-/-$ mice and all of the infected cells expressed $H\text{-}2K^b$ and a substantial fraction expressed A^b. In contrast, in $\beta_2M-/-$ mice, there were higher levels of *L. monocytogenes* at day 7 and nearly all of the infected splenocytes were class II-negative (Fig. 8b).

DISCUSSION

Our data, in accordance with recently published data from two other investigators,[28,29] demonstrate that $\beta_2M-/-$ knock-out mice have an impaired ability to clear promptly an infection with *L. monocytogenes*. Additionally, our data show several novel findings regarding the function of class II-restricted CTL as compared to class I-restricted CTL during a bacterial infection. Class II-restricted CTL lines, though effective in protection prior to infection with *L. monocytogenes*, do not

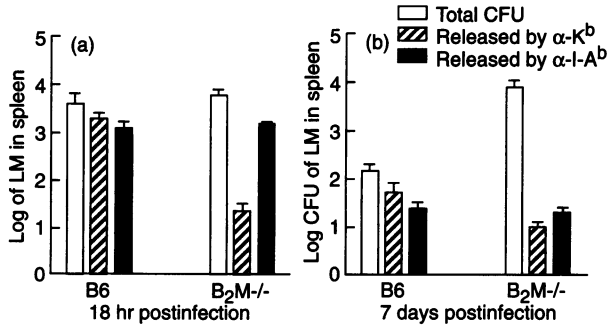

Figure 8 (a). Phenotype of splenocytes 18 hr after infection with *L. monocytogenes*. β_2M−/− and C57BL/6 mice were infected i.v. with 0·3 × the LD_{50} of *L. monocytogenes* for each type respectively ($1{\cdot}5 \times 10^3$ and 5×10^3 respectively). Eighteen hours later the animals were killed. Splenic homogenates were aliquoted evenly and treated with antibodies 7.18.3 (IgM reactive with I-A^b), 11.3.1 (IgM reactive with K^b) for 45 min at 4°. In addition, aliquots were treated with phosphate buffered saline/0·1% Triton-X-100 or media alone. Following antibody treatment, the splenocytes were treated with baby rabbit complement (1 : 10 dilution) at 37° for 30 min. The process was repeated × 1 and the supernatants collected and plated. Splenocytes that had not lysed with antibody treatment were then treated with PBS/0·1% Triton-X-100. >90% of the organisms in the β_2M−/− mice as assessed by the initial CFU from detergent lysis were recovered with this final treatment. Efficacy of lysis was assessed by flow cytometry on antibody-treated splenocytes using antibodies 7.16.17 and 28-8-6s. Data is presented as the mean of the log of CFU in the spleen with standard error bars. $n = 5$ mice for C57BL/6 and β_2M−/− groups.

Phenotype of splenocytes 7 days after infection with L. monocytogenes. Experiments were performed as described above. C57BL/6 and β_2M−/− mice were infected with 0·5 × LD_{50} for *L. monocytogenes* respectively and 7 days later the animals killed and the spleens removed and treated similarly. Data is presented as mean with standard error bars. $n = 5$ mice for C57BL/6 and β_2M−/− groups.

decrease the number of *L. monocytogenes* in the spleen or liver when administered as early as 24 hr after infection. The production of IFN-γ is not essential in the adoptive transfer of protection mediated by $CD4^+$ CTL. Even in the absence of detectable H-2K^b on the surface of cells, K^b-restricted CTL from C57BL/6 mice are able to reduce the numbers of *L. monocytogenes* when given at both 18 hr and 7 days postinfection. The persistence of infection in β_2M−/− mice is partly related to the inability of the immune response to eliminate organisms in class II-negative cells.

Roberts *et al.*[28] showed that $CD4^+$ bulk splenocytes from β_2M−/− mice were not capable of protecting C57BL/6 mice from a lethal infection with *L. monocytogenes*. We have presented data that $CD4^+$ CTL from lines stimulated *in vitro* can protect β_2M−/− mice. The CTL we transferred were enriched in number and specificity by stimulation with antigen. The difference in protection mediated by these cell lines is most likely due to the presence of a greater number of *L. monocytogenes*-specific CTL. Alternatively, there may be differences in the adoptive transfer of protection by $CD4^+$ lymphocytes in C57BL/6 versus β_2M−/− mice. Unlike the data presented by Roberts *et al.*, we have not been able to detect $CD8^+$ T lymphocytes in the spleens of β_2M−/− mice after infection with *L. monocytogenes*.

Harty and Bevan previously had demonstrated that the adoptive transfer of protection using $CD8^+$ CTL was not mediated by the production IFN-γ.[30] We now show that the adoptive transfer of protection of $CD4^+$ CTL is not dependent on the production of IFN-γ. These data are the first to demonstrate that the adoptive transfer of protection by $CD4^+$ CTL is independent of the production of IFN-γ.

We were surprised at the failure to decrease a bacterial load in an ongoing infection by the administration of I-A^b-restricted CTL. One explanation for the success of the adoptive transfer of CTL, prior to, but not after an infection, may relate to the method of spread of *L. monocytogenes* in the spleen. *L. monocytogenes* preferentially infects macrophages in the spleen. The organism polymerizes cellular actin via the interaction of the *actA* gene with the cellular actin machinery and this allows for the spread of the infection to adjacent cells without the need for the bacteria to be exposed to the extracellular environment.[31,32] We show that 18 hr after infection with *L. monocytogenes*, I-A^b-restricted CTL had a modest effect on the titres of bacteria in the spleen when given i.v. in adoptive transfer experiments. The limited expression of class II on target cells may explain the persistence of infection by pathogens that specifically elicit a class II-restricted response such as *L. monocytogenes* in this model or Herpes simplex.

The ability of K^b-restricted $CD8^+$ CTL to protect against a persistent or lethal challenge with *L. monocytogenes* was somewhat unexpected in the β_2M−/− mice. Lehmann-Grube *et al.*[33] had demonstrated that the transfer of splenocytes from previously infected B10.A(5R) (H-2 K^{b}A^{b}E^{k}D^d) mice could decrease the titres of lymphocytic choriomeningitis virus (LCMV) in H-2^b β_2M−/− mice. This effect was 100-fold less efficient than the transfer of H-2K^b,D^b splenocytes. However, other investigators have been unable to demonstrate an H-2K^b-restricted response to LCMV during a central nervous system infection.[34] These data questioned the presence of a K^b-restricted epitope in LCMV. We have confirmed that H-2K^b-restricted CTL can protect mice lacking β_2M suggesting the presence of a form of K^b on the surface of cells independent of β_2M.

In conclusion we have demonstrated that in β_2M−/− mice, I-A^b-restricted *L. monocytogenes*-specific killers can be generated. These cells, though functional in adoptive transfer experiments, are unable to clear promptly *L. monocytogenes* from the spleen. The delayed clearance of *L. monocytogenes* involves the persistence of infection in class II-negative cells in the spleen. We could alter both the delayed clearance of *L. monocytogenes* and a lethal challenge with *L. monocytogenes* by the administration of $CD8^+$ CTL from C57BL/6 mice. These experiments show that pathogens that do not elicit a class I-restricted response have evolved a strategy for escape from prompt elimination by the immune system despite the presence of cytolytic class II-restricted lymphocytes.

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