An MHC Class Ib-Restricted CD8\(^+\) T Cell Response to Lymphocytic Choriomeningitis Virus

Lili Chen, David C. Jay, Jared D. Fairbanks, Xiao He and Peter E. Jensen

*J Immunol* 2011; 187:6463-6472; Prepublished online 14 November 2011; doi: 10.4049/jimmunol.1101171

http://www.jimmunol.org/content/187/12/6463

**References**

This article cites 62 articles, 35 of which you can access for free at:

http://www.jimmunol.org/content/187/12/6463.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
An MHC Class Ib-Restricted CD8+ T Cell Response to Lymphocytic Choriomeningitis Virus

Lili Chen,1 David C. Jay,1 Jared D. Fairbanks, Xiao He, and Peter E. Jensen

Conventional MHC class Ia-restricted CD8+ T cells play a dominant role in the host response to virus infections, but recent studies indicate that T cells with specificity for nonclassical MHC class Ib molecules may also participate in host defense. To investigate the potential role of class Ib molecules in anti-viral immune responses, Kb−/−Dd−/−CIITA−/− mice lacking expression of MHC class Ia and class II molecules were infected with lymphocytic choriomeningitis virus (LCMV). These animals have a large class Ib-selected CD8+ T cell population and they were observed to mediate partial (but incomplete) virus clearance during acute LCMV infection as compared with Kb−/−Dd−/−β2-microglobulin−/− mice that lack expression of both MHC class Ia and class Ib molecules. Infection was associated with expansion of splenic CD8+ T cells and induction of granzyme B and IFN-γ effector molecules in CD8+ T cells. Partial virus clearance was dependent on CD8+ cells. In vitro T cell restimulation assays demonstrated induction of a population of β2-microglobulin–dependent, MHC class Ib-restricted CD8+ T cells with specificity for viral Ags and yet to be defined nonclassical MHC molecules. MHC class Ib-restricted CD8+ T cell responses were also observed after infection of Kb−/−Dd−/− mice despite the low number of CD8+ T cells in these animals. Long-term infection studies demonstrated chronic infection and gradual depletion of CD8+ T cells in Kb−/−Dd−/−CIITA−/− mice, demonstrating that class Ia molecules are required for viral clearance. These findings demonstrate that class Ib-restricted CD8+ T cells have the potential to participate in the host immune response to LCMV. The Journal of Immunology, 2011, 187: 6463–6472.

The mouse genome encodes a large number of nonclassical MHC class I (class Ib) molecules, including many with unknown functions (1, 2). Most mouse MHC class Ib genes are encoded at the H2-Q, T, and M region at the telomeric end of the MHC region (1). In general, class Ib genes show little polymorphism relative to the highly polymorphic classical MHC class I genes, H2-K, -D, and -L. Protein folding and expression in most cases requires assembly with β2-microglobulin (β2m). The MHC class Ib tissue distribution and expression levels vary considerably (3). Most class Ib molecules have a lower expression level on the cell surface compared with class Ia molecules. Nevertheless, a population of CD8+ T cells can be selected by the class Ib molecules expressed on hematopoietic cells and thymic epithelial cells in the thymus; the class Ib-restricted CD8+ T cells selected by the hematopoietic cells tend to have a more activated phenotype (4, 5). Some class Ib proteins, including CD1d and MR1, are encoded outside of the MHC. CD1d presents lipid Ags to CD8-negative NKT cells (6–8). MR1 controls the development and activation of cells in a commensal bacteria-dependent way, probably through presentation of yet undefined ligands (9, 10). NKt and mucosal-associated invariant T cells express highly restricted TCR repertoires with invariant TCRα-chains and restricted TCRβ repertoires and they have a capacity to be stimulated rapidly to produce cytokines through a “memory-like” response.

The H2-Q, -T, and -M regions of C57BL/6 (B6) mice encode >20 predicted protein-coding class Ib molecules. Several of these have been shown to function in the immune system. Qa-1 (encoded by H2-T23) regulates NK cell activation by serving as the ligand for the CD94/NKG2A inhibitory receptor (11–13). This class Ib molecule predominantly assembles with a peptide derived from the leader sequence of class Ia molecules, H2-D and L. However, Qa-1 also has the capacity to present a variety of self and foreign peptides to TCRαβ T cells (14–18). Qa-2 (encoded by H2-Q6, -Q7, -Q8, and -Q9) assembles with a highly diverse repertoire of endogenous peptides sharing a common peptide-binding motif (19) and functions as an Ag-presenting molecule in tumor immunity (20). H2-M3 selectively binds and presents N-formylated bacterial and mitochondrial peptides to CD8+ T cells (2, 21).

Various studies have implicated class Ib-restricted CD8+ T cells in the host response to intracellular bacterial pathogens. Both Qa-1−/− and H2-M3–restricted T cell responses can be demonstrated in mice postinfection with Listeria monocytogenes (22–26). The H2-M3–restricted T cell response to L. monocytogenes is characterized by a rapid primary response and relatively little memory response (27, 28). Impaired early bacterial clearance in H2-M3–deficient mice demonstrates a nonredundant role for H2-M3 in L. monocytogenes infection (29). The higher bacterial burden in Kb−/−Dd−/−M3−/− mice compared with Kb−/−Dd−/− mice at day 7 after L. monocytogenes infection suggests that H2-M3 has protective function against L. monocytogenes infection (30). H2-M3–restricted T cell responses can also be demonstrated in Chlamydia pneumoniae, Mycobacterium tuberculosis, and Salmonella enterica infections (31–34).

Recently, evidence has been published demonstrating that class Ib-restricted CD8+ T cells can also participate in the immune response to viruses. Braaten et al. (35) demonstrated that class Ib-
restricted CD8+ T cells effectively control chronic γ-herpesvirus 68 (γHV68) infections in class Ia-deficient K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/ mice. Virus infection was not controlled in β\(_{\text{m}}\)-deficient animals, whereas it was controlled equivalently in B6 and B6.K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/ mice. Although the class Ib specificit... to that observed in B6 mice. The T cell response was observed to be primarily focused on a nonamer peptide from the VP2 capsid protein containing the previously defined Qa-2 peptide-binding motif. These two studies raise the possibility that class Ib molecules might participate in the host response to other virus infections.

A relatively small number of CD8+ T cells are selected by class Ib molecules in the thymus, and these cells are prevented from undergoing extensive homeostatic expansion in peripheral lymphoid organs by a numerical excess of conventional class Ia- and class II-restricted T cells. However, class Ib-restricted T cells expand to relatively large populations in the secondary lymphoid organs of K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/CIITA\(^{-}\) mice that lack conventional T cells selected by MHC class Ia or class II molecules (37). These mice provide an interesting model to study the potential role of class Ib-restricted T cells in virus infections. Lymphocytic choriomeningitis virus (LCMV) is a prototype arenavirus that has been studied extensively in its natural rodent host to investigate immune responses in the setting of acute or chronic virus infection. Viral clearance in acute LCMV Armstrong infections is dependent on CD8+ T cells and β\(_{\text{m}}\) (38). H2-D\(^{\text{d}}\)- and K\(^{\text{b}}\)-restricted CD8+ T cells with defined LCMV peptide specificity markedly expand during acute infection to represent ≥70% of the total CD8+ T cells in the spleen at day 8 postinfection in B6 mice (39). In the present study, the T cell response to acute LCMV Armstrong infection was evaluated in K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/CIITA\(^{-}\) and K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/ mice. The results demonstrate that LCMV can elicit β\(_{\text{m}}\)-dependent, class Ib-restricted CD8+ T cells that have a capacity to mediate partial viral clearance during the acute phase of virus infection. However, these T cells are insufficient to clear virus and prevent chronic virus infection.

**Materials and Methods**

**Mice**

B6, CIITA\(^{-}\)/, β\(_{\text{m}}\)-/ mice, and B6.K1(Qa-2\(^{-}\)/) mice were obtained from The Jackson Laboratory. The CIITA\(^{-}\)/ mice were backcrossed with B6 mice for seven generations (The Jackson Laboratory). K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/ mice were backcrossed to B6 mice for 10 generations and were obtained from Taconic Farms (40). K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/CIITA\(^{-}\)/ mice and K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/β\(_{\text{m}}\)-/ mice were generated by crossing K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/ mice with either CIITA\(^{-}\)/ mice or β\(_{\text{m}}\)-/ mice (37). B6.129S6-H2-T2.2\(^{\text{m}}\text{CIC}^{-}\)/ mice, which are Qa-1+/−, were a gift from Dr. Harvey Cantor (Harvard Medical School). CD1d\(^{-}\)/ mice were a gift from Dr. Ted Hansen (Washington University School of Medicine). H2-M3+/− mice were a gift from Dr. Chyung-Ru Wang (Northwestern University). Eight- to 12-wk-old mice were used in the experiments. Both sexes of mice were used in the LCMV clearance assay; the mice were sex-matched in other experiments. Mice were housed in a specific pathogen-free facility at the University of Utah and were handled according to the Institutional Animal Care and Use Committee policies.

**Cell lines**

Vero cells and BHK-21 cells were cultured in DMEM media supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml l-glutamine (Invitrogen). Mouse primary cells were cultured in RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml l-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 55 μM 2-ME (Invitrogen). All of the cells were maintained in a humidified 37°C, 5% CO\(_2\) incubator.

**Virus and infection**

LCMV Armstrong virus was a gift from Dr. Matthew Williams (University of Utah). Virus was maintained in BHK-21 cells, and the titer was obtained by Vero cell plaque assay (41). Briefly, 6 × 10\(^5\) Vero cells per well were seeded in a six-well plate (Corning Costar) 1 d before the plaque assay so that they would form confluent monolayers. Then, virus stocks were 10-fold serially diluted in DMEM media, and 200 μl diluted virus was added on top of the Vero cell monolayer, after which the plate was incubated for 1 h before the addition of 0.5% agarose in Media 199 (Invitrogen). Four days later, 0.5% agarose in Media 199 containing neutral red was added to the plate, and the plaque number was counted the following day. The infection of mice was achieved by i.p. inoculation with LCMV Armstrong virus in a dose of 2 × 10\(^5\) PFU/mouse. At certain time points after the infection, mouse tissues were harvested for the desired experiments. Tissues collected for plaque assay were weighed and homogenized in DMEM media, followed by 10-fold serial dilutions and incubation with Vero cell monolayers to determine virus titers as described (41).

**Abs and flow cytometry**

Mouse-specific TCRβ (H57-597), CD4 (GK1.5, RM4-5), CD8α (53-6.7), IFN-γ (XMG1.2), granzyme B (16G6) mAbs, and TCR Vβ panel Abs conjugated with FITC, PE, PerCP, allophycocyanin, PerCP-Cy5.5, PE-Cy5, PE-Cy7, Pacific Blue, or Alexa Fluor 488 fluorophore were purchased from BD Biosciences or eBioscience. Suspended single cells were stained with Abs for detecting surface proteins in 4°C for 20 min. If necessary, intracellular staining was done after surface staining by the BD Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences) according to the manufacturer’s instructions. Cells were fixed by 1% PFA after staining. The fluorescence was detected by a FACS Canto II machine (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star).

**CD8+ T cell depletion**

Mice were i.p. injected with 300 μg CD8 mAb (2-43) at days 3, 2, and 1 before the LCMV infection and at day 6 after the LCMV infection. The depletion efficiency was ≥95% based on analysis by flow cytometry.

**In vitro restimulation assay**

Peritoneal macrophages or bone marrow-derived macrophages were used as APCs in the assay. To harvest the peritoneal macrophages, mice were i.p. injected with 100 μg Con A; 3 d after the injection, peritoneal exudates were harvested and 4 × 10\(^5\) cells were plated in one well of the 96-well plate; 2 h after incubation, nonadherent cells were washed away and the remaining macrophage cells were either mock treated or infected with LCMV Armstrong for 1 d. Bone marrow cells were harvested from the femur and tibia and cultured in media containing L929 supernatant for 6 d; the nonadherent cells were removed and the remaining adherent cells, which were >99% F4/80+, were harvested and replated in 1 × 10\(^5\) cells per well as APCs; the cells were either mock treated or infected with LCMV Armstrong for 1 d before the addition of responding cells. Spleens from LCMV-infected mice were harvested at day 8 after the LCMV infection. Single splenocyte suspensions were made, and CD8+ T cells were enriched using a CD8α+ T cell isolation kit (Miltenyi Biotec) and further purified by staining with CD8 Ab (53-6.7), followed by FACS sorting to get highly purified CD8+ T cells, if necessary. Enriched CD8+ T cells (1 × 10\(^5\)) were added as responding cells to the prepared APC presenting macrophages at 5 μg/ml 1 h before the addition of the responding cells. The cells were cocultured for 20 h, 10 μg/ml brefeldin A was added to the cells for an additional 4 h, and the cells were harvested for analysis of IFN-γ production by flow cytometry.

**Results**

Partial clearance of LCMV Armstrong in mice lacking conventional MHC class Ia and class II molecules

Previous studies have demonstrated that CD8+ T cells and β\(_{\text{m}}\) are required to clear virus during acute LCMV Armstrong infections (38). In B6 mice, the CD8+ T cell response is predominantly focused on three immunodominant peptides presented by H2-D\(^{\text{d}}\) and an additional epitope presented by H2-K\(^{\text{b}}\) (39). We used K\(^{\text{b}}\)-/D\(^{\text{d}}\)-/CIITA\(^{-}\)/ mice to evaluate the capacity of MHC class Ib-restricted...
T cells to recognize LCMV Ags and substitute for conventional CD8+ T cells in controlling acute infection. Kb-b2-Ddb-CIITA-/mice, which express class Ib molecules but not class Ia or class II MHC proteins (37), were observed to have lower virus burden and to partially clear LCMV by day 8 postinfection as compared with Kb-b2-Ddb-/βm+/mice, which lack expression of both class Ib and class Ia molecules (Fig. 1). Viral titers in the spleens of Kb-b2-Ddb-/CIITA-/mice were generally reduced 2–3 logs as compared with βm-deficient animals. In contrast, B6 mice consistently cleared virus to undetectable levels at day 8 or 12 postinfection. These results raised the possibility that T cells with specificity for class Ib molecules may have a capacity to contribute to the host immune response in acute LCMV infection.

Expansion of CD8+ TCRαβ T cells during acute viral infection
Kb-b2-Ddb-/CIITA-/mice have CD1d-restricted NKT cells, in addition to an expanded population of CD8+ T cells with specificity for class Ib molecules. Similar to B6 mice, an expansion in the fraction of CD8+ T cells in the spleen was observed in class Ia-deficient mice during acute LCMV infection (Fig. 2A, 2B), but the total number of CD8+ T cells per spleen was considerably greater in B6 animals before and after infection. CD8+ T cells from class Ia-deficient mice express a diverse TCR repertoire as judged by Vβ-chain usage (37, 42). A similarly diverse Vβ repertoire was observed in the CD8+ T cell populations in the spleens of Kb-b2-Ddb-/CIITA-/mice 8 d postinfection (Fig. 2C). However, the postinfection repertoire was skewed as compared with uninfected animals, with increases in the fraction of CD8+ T cells expressing Vβ8, Vβ10, and Vβ13. Despite undetectable expression of MHC class II molecules in Kb-b2-Ddb-/CIITA-/mice, a substantial population of CD4+ T cells is present in the peripheral lymphoid organs in these animals (42). As with the CD8+ T cells in these mice, it is likely that the CD4+ T cells have a history of homeostatic expansion.

A substantial fraction of the CD4+ T cells appears to represent NKT cells with specificity for CD1d (data not shown). No expansion of CD4+ T cells was observed in Kb-b2-Ddb-/CIITA-/mice after acute LCMV infection. Indeed, the absolute number for CD4+ T cells was decreased in spleens postinfection (Fig. 2D), which may reflect a reduced number of CD1d-restricted NKT cells upon acute LCMV infection (43, 44). Thus, CD8+ T cells selectively undergo expansion during acute LCMV infection of Kb-b2-Ddb-/CIITA-/mice.

Effector phenotype in virus-expanded CD8+ T cells
Most of the CD8+ T cells in naive Kb-b2-Ddb-/CIITA-/mice have a memory-like surface phenotype, CD44high, Ly6chigh, β7 integrinlow, and CD122high, reflecting a history of homeostatic expansion (37). This phenotype makes it complicated to evaluate the T cells for effector differentiation postinfection. To assess the differentiation state of the virus-expanded CD8+ T cells in Kb-b2-Ddb-/CIITA-/mice, we used granzyme B expression as a marker of cells with effector function (45, 46). During the course of an acute LCMV Armstrong infection, CD8 T cells from Kb-b2-Ddb-/CIITA-/mice robustly produced granzyme B throughout the infection, and the fraction of CD8+ T cells expressing granzyme B was greater than or equal to that observed in B6 mice (Fig. 3A). No granzyme B expression was observed in CD8+ T cells from Kb-b2-Ddb-/CIITA-/mice prior to infection. The expression level in individual cells exceeded that observed in CD8+ T cells from B6 mice as measured by mean fluorescence intensity in flow cytometric analysis at several time points (Fig. 3B). These data suggest that most CD8+ T cells in Kb-b2-Ddb-/CIITA-/mice acquire effector function early in the course of LCMV Armstrong infection and this is sustained for at least 12 d postinfection.

As an additional indicator of effector function, ex vivo production of IFN-γ by CD8+ T cells in response to LCMV infection was assessed. At various time points postinfection, B6 and Kb-b2-Ddb-/CIITA-/mice splenocytes were harvested and permeabilized to evaluate the production of IFN-γ. A fraction of CD8+ T cells in Kb-b2-Ddb-/CIITA-/mice produced IFN-γ by day 5 after LCMV infection (Fig. 4). Whereas the peak of the B6 CD8+ T cell IFN-γ response was observed at day 5 postinfection, the fraction of IFN-γ producing CD8+ T cells continued to expand during the time points analyzed in Kb-b2-Ddb-/CIITA-/mice. Similar results were observed with animals injected with brefeldin A 6 h prior to analysis to inhibit cellular cytokine secretion (47) (data not shown). The continued production of IFN-γ in these animals might reflect an ongoing response to viral Ags given that these animals do not fully clear infection. Overall, the results indicate that CD8+ T cells in Kb-b2-Ddb-/CIITA-/mice acquire effector functions during acute LCMV infection.

Requirement for CD8+ cells in controlling virus replication in Kb-b2-Ddb-/CIITA-/mice
Depletion experiments were performed to evaluate the role of CD8+ T cells in controlling acute LCMV infection in Kb-b2-Ddb-/CIITA-/mice. Mice were injected with CD8 Ab specific for the CD8α heterodimer to selectively deplete CD8αβ T cells but not CD8α-expressing dendritic cells or CD8αα-expressing CD8 T cells. Depletion was >95% in these experiments (data not shown). Based on plaque-forming assays, virus was cleared to undetectable levels in the spleen, liver, kidney, and lung of B6 mice at day 8 postinfection (Fig. 5). As observed previously, virus was still present in the spleens of Kb-b2-Ddb-/CIITA-/mice, but titers were reduced 2–3 logs relative to Kb-b2-Ddb-/βm+/mice. In other tissues, virus was reduced to undetectable levels in Kb-b2-Ddb-/CIITA-/ but not in Kb-b2-Ddb-/βm-/mice. Depletion of CD8+ T cells resulted in complete reversal of virus clearance in B6 and Kb-b2-Ddb-/CIITA-/mice. Titers in the organs of CD8-depleted animals were similar to those observed in Kb-b2-Ddb-/βm+/mice. These results suggest that CD8+ T cells are essential for the partial viral clearance observed in mice lacking conventional MHC class Ia- and class II-restricted T cells.

Direct evidence for a class Ib-restricted CD8+ T cell response to LCMV
Under certain conditions, granzyme B and IFN-γ production can be induced directly in memory phenotype CD8+ T cells by cytokines such as IL-15 in the absence of TCR-mediated Ag recog-
nition (48). Given the memory-like phenotype of CD8+ T cells in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice, we could not exclude the possibility that expansion of CD8+ T cells and acquisition of effector functions were induced through nonspecific bystander mechanisms as opposed to direct Ag recognition by class Ib-restricted T cells. In vitro assays were performed to investigate the specificity of CD8+ T cells from LCMV-infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. Initial experiments demonstrated that CD8+ T cells from LCMV-infected B6 mice produce IFN-\gamma during 6 h coculture assays with LCMV-infected syngeneic macrophages, measured by intracellular cytokine staining and flow cytometry. However, under identical conditions, no response was observed in T cells from LCMV-infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice (data not shown). A variety of experimental parameters were analyzed and we found that in vitro responses were observed when the culture period was extended. A large fraction of CD8+ T cells purified from 8 d postinfection K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice produces IFN-\gamma after a 24-h culture with LCMV-infected but not uninfected syngeneic macrophages (Fig. 6A). LCMV-immune CD8+ T cells from B6 mice respond in vitro to infected macrophages from B6 but not K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} or K\textsuperscript{b/-}/D\textsuperscript{b/-}/\beta\textsubscript{m/-/-} mice, indicating that the T cell response in these animals is dominated by class Ia-restricted T cells. In contrast, CD8+ T cells from infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice respond equally well to infected peritoneal macrophages from B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. However, they do not respond to infected macrophages from K\textsuperscript{b/-}/D\textsuperscript{b/-}/\beta\textsubscript{m/-/-} mice. These results provide direct evidence that LCMV infection induces a population of Ag-specific, \beta\textsubscript{m}-

Response of K\textsuperscript{b/-}/D\textsuperscript{b/-} mice to the LCMV infection

K\textsuperscript{b/-}/D\textsuperscript{b/-} mice, deficient in MHC class Ia but not class II molecules, have normal numbers of CD4+ T cells and very few CD8+ T cells. Although few in number, the CD8+ T cells in these animals are thought to be selected to by MHC class Ib molecules, and we were interested in determining whether a class Ib-restricted T cell response would be observed after LCMV infection. Indeed, the CD8+ T cells from day 8 infected K\textsuperscript{b/-}/D\textsuperscript{b/-} mice responded to syngeneic LCMV-infected macrophages, but
Chronic virus infection in K\textsuperscript{b/}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice

Analysis of the acute phase of virus infection demonstrated that substantial titers of virus were present in spleen at day 8 (and 12) postinfection in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. Long-term infection experiments were performed to determine whether virus was eventually cleared in these animals. As shown in Fig. 8A, continued viral replication occurs after the initial period of infection such that very high viral titers are present at day 37, similar to titers present at day 3 postinfection. Thus the class Ib-restricted CD8\textsuperscript{+} T cells in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice are insufficient to clear LCMV, and a state of chronic infection is established. The vast majority of chronically infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice survived for at least 80 d. However, spleen CD8\textsuperscript{+} T cells and IFN-γ-producing T cells continuously declined in number (Fig. 8B, 8C) such that they were almost undetectable by day 82 postinfection.

3. Analysis of the acute phase of virus infection demonstrated that substantial titers of virus were present in spleen at day 8 (and 12) postinfection in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. Long-term infection experiments were performed to determine whether virus was eventually cleared in these animals. As shown in Fig. 8A, continued viral replication occurs after the initial period of infection such that very high viral titers are present at day 37, similar to titers present at day 3 postinfection. Thus the class Ib-restricted CD8\textsuperscript{+} T cells in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice are insufficient to clear LCMV, and a state of chronic infection is established. The vast majority of chronically infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice survived for at least 80 d. However, spleen CD8\textsuperscript{+} T cells and IFN-γ-producing T cells continuously declined in number (Fig. 8B, 8C) such that they were almost undetectable by day 82 postinfection.

FIGURE 3. Early and robust LCMV-specific granzyme B production by CD8 T cells during LCMV infection in K\textsuperscript{b/}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. Splenocytes from LCMV-infected B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice were stained on the surface for CD8 and TCR, then intracellularly for granzyme B and analyzed by flow cytometry. A. The percentage of granzyme B-producing CD8\textsuperscript{+} TCR\textsuperscript{+} splenocytes from B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice at days 3, 5, 8, and 12 after LCMV Armstrong infection. B. The mean fluorescence intensity (MFI) of granzyme B expression on the CD8\textsuperscript{+} T cells during LCMV infection in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice.

FIGURE 4. Substantial ex vivo IFN-γ production by CD8\textsuperscript{+} cells from LCMV-infected mice. A. B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice were infected with 2 \times 10\textsuperscript{5} PFU LCMV Armstrong and, at days 3, 5, 8, and 12, spleens were harvested and analyzed for surface CD8\textsuperscript{+} and intracellular IFN-γ by flow cytometry. B. The percentage and total number of IFN-γ-producing CD8\textsuperscript{+} spleen lymphocytes was determined for B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice at various time points postinfection.

not to the K\textsuperscript{b/-}/D\textsuperscript{b/-}/β\textsubscript{2}m\textsuperscript{-/-} macrophages (Fig. 7A). Consistent with a specific immune response, the fraction and absolute number of spleen CD8\textsuperscript{+} T cells expanded after acute LCMV infection (Fig. 7B). A marked expansion of CD8\textsuperscript{+} T cells with effector phenotype was also observed as measured by ex vivo IFN-γ production (Fig. 7C) and granzyme B (data not shown).

Chronic virus infection in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice

Analysis of the acute phase of virus infection demonstrated that substantial titers of virus were present in spleen at day 8 (and 12) postinfection in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice. Long-term infection experiments were performed to determine whether virus was eventually cleared in these animals. As shown in Fig. 8A, continued viral replication occurs after the initial period of infection such that very high viral titers are present at day 37, similar to titers present at day 3 postinfection. Thus the class Ib-restricted CD8\textsuperscript{+} T cells in K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice are insufficient to clear LCMV, and a state of chronic infection is established. The vast majority of chronically infected K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice survived for at least 80 d. However, spleen CD8\textsuperscript{+} T cells and IFN-γ-producing T cells continuously declined in number (Fig. 8B, 8C) such that they were almost undetectable by day 82 postinfection.

FIGURE 5. Control of LCMV Armstrong infection in B6 and K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-} mice is dependent on CD8\textsuperscript{+} cells. B6, K\textsuperscript{b/-}/D\textsuperscript{b/-}/CIITA\textsuperscript{-/-}, and K\textsuperscript{b/-}/D\textsuperscript{b/-}/β\textsubscript{2}m\textsuperscript{-/-} mice were either mock treated or i.p. inoculated with CD8-specific Ab (clone 2.43) at days −3, −2, and −1 and day 6 of LCMV Armstrong infection to deplete the CD8\textsuperscript{+} T cells. Spleen, liver, kidney, and lung tissues were harvested for plaque assay at day 8 after LCMV Armstrong infection.
A Peritoneal macrophages were harvested from Con A-stimulated B6, K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), or K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\) mice as a source of APCs. APCs were either uninfected or infected with LCMV Armstrong for 1 d. At day 8 after LCMV infection, splenocytes were harvested from B6 or K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice and CD8\(^{+}\) T cells were enriched and cocultured with APCs for 1 d, followed by analysis of IFN-\(\gamma\) production by flow cytometry. Left, Representative flow cytometry plots showing the production of IFN-\(\gamma\) by cocultured CD8\(^{+}\) T cells. Right, Percentage of IFN-\(\gamma\)-producing CD8\(^{+}\) T cells. T cells were obtained from three mice for each group. The data represent one of three independent experiments.

B, Six K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\), Qa-1/-, B6.K1(Qa2/-), and CD1d\(^{-/-}\) bone marrow-derived macrophages were used as APCs. CD8\(^{+}\) T cells enriched from day 8 infected mice were cocultured with the LCMV uninfected or infected APCs for 1 d, and the production of IFN-\(\gamma\) was measure by flow cytometry. C, B6, K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\), and H2-M3\(^{-/-}\) bone marrow-derived macrophages were used as APCs. MR1 blocking Ab was added as indicated to the B6 or the K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) APCs in 5 \(\mu\)g/ml to block the MR1 molecule. CD8\(^{+}\) T cells enriched from day 8 infected mice were cocultured with the LCMV uninfected or infected APCs for 1 d, and the production of IFN-\(\gamma\) was measure by flow cytometry.

**FIGURE 6.** MHC class Ib-restricted CD8\(^{+}\) T cells from LCMV-infected K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice. A, Peritoneal macrophages were harvested from Con A-stimulated B6, K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), or K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\) mice as a source of APCs. APCs were either uninfected or infected with LCMV Armstrong for 1 d. At day 8 after LCMV infection, splenocytes were harvested from B6 or K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice and CD8\(^{+}\) T cells were enriched and cocultured with APCs for 1 d, followed by analysis of IFN-\(\gamma\) production by flow cytometry. Left, Representative flow cytometry plots showing the production of IFN-\(\gamma\) by cultured CD8\(^{+}\) T cells. Right, Percentage of IFN-\(\gamma\)-producing CD8\(^{+}\) T cells. T cells were obtained from three mice for each group. The data represent one of three independent experiments. B, Six K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\), Qa-1/-, B6.K1(Qa2/-), and CD1d\(^{-/-}\) bone marrow-derived macrophages were used as APCs. CD8\(^{+}\) T cells enriched from day 8 infected mice were cocultured with the LCMV uninfected or infected APCs for 1 d, and the production of IFN-\(\gamma\) was measure by flow cytometry. C, B6, K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\), K\(^{b/-}\)D\(^{b/-}\)\(\beta\_m/-\), and H2-M3\(^{-/-}\) bone marrow-derived macrophages were used as APCs. MR1 blocking Ab was added as indicated to the B6 or the K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) APCs in 5 \(\mu\)g/ml to block the MR1 molecule. CD8\(^{+}\) T cells enriched from day 8 infected mice were cocultured with the LCMV uninfected or infected APCs for 1 d, and the production of IFN-\(\gamma\) was measure by flow cytometry.

**Discussion**

In the present study, K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice lacking conventional class Ia and class II MHC molecules were used to investigate the potential for class Ib-selected T cells to mount an Ag-specific effector response to LCMV infection. These animals were observed to mediate partial but incomplete virus clearance during acute LCMV Armstrong infection. However, clearance was incomplete and a chronic infection followed. Infection was associated with an initial expansion of splenic CD8\(^{+}\) T cells and induction of granzyme B and IFN-\(\gamma\) effector molecules in this population of cells, followed by a continuous decline and ultimate depletion of CD8\(^{+}\) T cells. CD8 depletion experiments demonstrated that partial viral clearance in the early phases of infection was dependent on CD8alpha\(\beta\)\(\delta\) cells. In vitro T cell stimulation assays demonstrated the induction of a population of LCMV-specific, MHC class Ib-restricted CD8\(^{+}\) T cells in both K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) and K\(^{b/-}\)D\(^{b/-}\) animal strains.

The most definitive evidence for a class Ib-restricted T cell response to LCMV comes from in vitro assays demonstrating cytokine production by T cells cultured with LCMV-infected APCs. Ag presentation to T cells from K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) and K\(^{b/-}\)D\(^{b/-}\) mice required \(\beta\_m\) but not K\(^{b}\) or D\(^{b}\) class Ia molecules. This contrasts with the in vitro recall response of T cells from B6, which is clearly dominated by class Ia-restricted T cells, with very little response induced by K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) or K\(^{b/-}\)D\(^{b/-}\) APCs. A key element to establish an assay for detecting an in vitro response of LCMV-immune T cells from Kb\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice was the requirement for a longer culture period. Very strong IFN-\(\gamma\) responses were observed by intracellular staining after 6 h culture of CD8\(^{+}\) T cells from LCMV-immune B6 mice with infected APCs. Under the same conditions, no response was observed with T cells from K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice. However, a substantial fraction of T cells was observed to produce IFN-\(\gamma\) after 24 h culture. The delayed kinetics of cytokine production could reflect inherent differences in the function of class Ib- and class Ia-restricted T cells, delayed kinetics of class Ib ligand generation in infected APCs, or some form of early CD8\(^{+}\) T cell exhaustion in K\(^{b/-}\)D\(^{b/-}\)CIITA\(^{-/-}\) mice (49, 50), making the T cells more resistant to restimulation in vitro.
Kb/db-CIITA-/- mice have markedly expanded numbers of peripheral CD8+ T cells compared with Kb/db-Db/- mice because they lack conventional class II-restricted CD4+ T cells that compete for homeostatic factors and inhibit the peripheral expansion of the relatively small number of CD8+ T cells selected in the thymus by class Ib molecules in Kb/db-Db/- mice (37). CD8+ T cells in Kb/db-Db/-/CIITA-/- mice have a memory-like phenotype as a result of their history of extensive homeostatic expansion, and thus they may be more sensitive to stimulation in the setting of high viral replication may make this unconventional population of T cells more susceptible to exhaustion and depletion (52–54).

The H2-M3–restricted CD8+ T cell response in acute infection with L. monocytogenes is observed to occur more rapidly than the class Ia-restricted T cell response, and the class Ib-restricted T cells appear to comprise a major fraction of the responding T cell population during acute infection (21, 27). In contrast, memory development is defective in the H2-M3–restricted response, potentially a result of inefficient competition with class Ia-restricted T cells for interaction with dendritic cells during secondary infection (27, 28, 55). T cells with specificity for H2-M3 represent only a small fraction of total Ag-specific cells in recall responses. Thus, in this model, class Ib-restricted T cells appear to function as a bridge between innate and adaptive immunity in acute infection, with little contribution to immunological memory. In LCMV-infected Kb/db-Db/-/CIITA-/- mice, we observed early expression of granzyme B in CD8+ T cells. IFN-γ production in vivo appeared to have similar early kinetics relative to T cells from B6 mice, but with a progressive increase until at least day 12 postinfection, long after the disappearance of IFN-γ–producing T cells in B6 animals. The continued production of IFN-γ may reflect an ongoing T cell response to residual virus in the mutant mice. A determination of whether class Ib-restricted T cells participate in the early stages of the host response to LCMV in mice expressing a normal complement of MHC molecules will require identification of the specificity of the class Ib-restricted T cell response.

Braaten et al. (35) were first to report that class Ib-restricted CD8+ T cells can effectively control a chronic viral infection.
but not β2m-deficient mice were observed to control γHV68 infection through a mechanism dependent on TCRαβ+ CD8+ T cells. This was associated with a marked increase in CD8+ T cells in the spleen at day 42 postinfection. The expanded CD8+ T cells acquired an effector/memory phenotype based on cell surface phenotype and the capacity to produce IFN-γ and TNF-α after in vitro stimulation with PMA and ionomycin. A dramatic skewing the TCR Vβ repertoire of CD8+ T cells was observed in Kb−/− Db−/− mice after γHV68 infection, with 70% of cells expressing Vβ4. Despite highly restricted Vβ utilization, analysis of CDR3 length demonstrated considerable diversity in the responding T cell repertoire.

Analysis of TCR Vβ utilization in CD8+ T cells from LCMV-infected Kb−/− Db−/− Ciita−/− mice demonstrated some skewing in the Vβ repertoire as compared with infected or uninfected B6, Kb−/− Db−/− β2m−/− bone marrow-derived macrophages for 1 d, and the IFN-γ production was measured by flow cytometry.

The recent study by Swanson et al. (36) was the first to define the specificity of class Ib-restricted immune response to a virus. In contrast to β2m-deficient animals, Kb−/− Db−/− mice were observed to rarely develop tumors postinfection with oncogenic mouse PyV. Similar to class Ia-expressing B6 mice, Kb−/− Db−/− mice were able to clear acute PyV infection and to check persistent viral infection. In contrast to our results with LCMV, no appreciable increase in the number of CD8+ T cells was detected in spleens until 25 d postinfection. Indeed, Ag-specific T cells were observed to slowly accumulate until 30–70 d postinfection. The induction of IFN-γ production by in vitro peptide stimulation of polyclonal CD8+ T cells from PyV-infected Kb−/− Db−/− mice was difficult to demonstrate (36). It is possible that cytokine responses would be observed in this system after longer restimulation cultures, similar to our findings with class Ib-restricted T cells induced by LCMV. CD8+ T cell clones and MHC tetramers were used to demonstrate that the class Ib-restricted T cell response to PyV is highly focused on a single epitope from the VP2 capsid protein presented by Q9 (Qa-2). T cells with this specificity represented 40% of the CD8+ T cells in the spleen at day 80 postinfection. Despite a capacity to control PyV infection, the class Ib-restricted T cells showed a pronounced functional
improvement, possibly resulting from persistent infection, chronic stimulation, and partial CD8^+ T cell exhaustion.

The specificity of the class Ib-restricted CD8^+ T cell response to LCMV identified in the present study was studied using MHC class Ib-deficient mice and an MR1 blocking Ab. Ag-specific, class Ib-restricted CD8^+ T cell responses have been demonstrated for Qa-1, Qa-2, and H2-M3. The results using APCs from Qa-1-deficient donor mice suggest that Qa-1 does not play a significant role in this response. H2-M3, with its strong preference for binding N-formylated bacterial and mitochondrial peptides, seems an unlikely candidate for presentation of viral epitopes. Note, however, that recognition of a nonformylated influenza virus hemagglutinin peptide by H2-M3-restricted cytotoxic T cells has been described (56–58). Nevertheless, there was no significant reduction observed in the restimulation response when the K^b^/D^b^/H2-M3^+^/CD8^+^ T cells were cocultured with infected H2M3^+/^ APCs. Responses were slightly reduced with APCs from Qa-2-deficient B6.K1 mice, but we cannot rule out the potential impact of differential expression of other molecules in this case. No effect was observed with an MR1 blocking Ab, although we still cannot absolutely rule out participation of this molecule. The murine H2-Qa-1–Ib and -M regions of B6 mice encode >20 predicted protein-coding class Ib molecules. Several, including thymic leukemia Ag (TL, encoded by T3/T18 d) and T10/T22, do not bind peptides (59, 60). TL interacts with high affinity to CIITA and regulates the function of CD8^+ T cells (61). T22 is a ligand for a population of TCR^B^ T cells (60, 62). However, a large number of class Ib molecules with potential Ag presentation function remain to be characterized. Although class Ib-selected T cells represent a relatively small fraction of the total CD8^+ TCR^B^ repertoire, there is growing evidence for their potential participation in the immune response to a diverse array of microbial pathogens.

Acknowledgments

We are grateful to Dr. Matthew Williams for critical reagents and advice and Dr. Atilla Kumanovics for helpful discussions. We thank David Coe and Hu Dai for excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

restricted CD8+ T cells undergo homeostatic expansion in the absence of con
38. Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are re
quired to sustain CD8+ cytotoxic T-cell responses during chronic viral infection.
T cells: a reevaluation of bystander activation during viral infection. Immunity 8:
177–187.
40. Péramau, B., M. F. Saron, B. Reina San Martin, N. Bervas, H. Ong,
Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+
T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic respon
Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of
persistently infected mice: role in suppression of cytotoxic T lymphocyte re
42. Laouini, D., A. Casrouge, S. Dalle, F. Lemonnier, P. Kourilsky, and
J. Kanellopoulos. 2000. Vβ T cell repertoire of CD8+ splenocytes selected on
43. Hobbs, J. A., S. Cho, T. J. Roberts, V. Sriram, J. Zhang, M. Xu, and
following infection with lymphocytic choriomeningitis virus. J. Virol. 75:
10746–10754.
term loss of canonical NKT cells following an acute virus infection. Eur. J.
225.
46. Bannard, O., M. Kraman, and D. Fearon. 2009. Pathways of memory CD8+
47. Liu, F., and J. L. Whitton. 2005. Cutting edge: re-evaluating the in vivo cytokine
response of CD8+ T cells during primary and secondary viral infections. J.
T cell receptor crosslinking in the induction of cellular proliferation, gene ex
pression, and cytotoxicity in CD8+ memory T cells. Proc. Natl. Acad. Sci. USA
99: 6192–6197.
49. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia,
signature of CD8+ T cell exhaustion during chronic viral infection. Immunity 27:
670–684.
J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of
activated T cells without effector function. J. Exp. Med. 188: 2205–2213.
specific NK1 T cells: development, specificity, and function. Annu. Rev.
is dictated by antigen exposure history and nature of the infection. Immunity
34: 781–793.
H2-M3-restricted memory T cells: persistence and activation without expansion.
ventional (TL-encoded) major histocompatibility complex molecules present
3028.
59. Weber, D. A., A. Attinger, C. C. Kemball, J. L. Wigal, J. Pohl, Y. Xiong,
E. L. Reinherz, H. Cheroutre, M. Kronenberg, and P. E. Jensen. 2002. Peptide-
dependent folding and CD8aa binding by the nonclassical class I molecule,
60. Schil, H., N. Mavaddat, C. Litzenberger, E. W. Ehrich, M. M. Davis,
61. Leishman, A. J., O. V. Naidenko, A. Attinger, F. Koning, C. J. Lena, Y. Xiong,
responses modulated through interaction between CD8αα and the nonclassical