T-cell intrinsic expression of MyD88 is required for sustained expansion of the virus-specific $CD8^+$ T-cell population in LCMV-infected mice

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Acute infection with lymphocytic choriomeningitis virus (LCMV) normally results in robust clonal expansion of virus-specific $CD8^+$ T cells, which in turn control the primary infection. However, similar infection of myeloid differentiation factor 88 (MyD88)-deficient mice leads to a markedly impaired T-cell response and chronic infection. It has been found previously that impairment of the innate immune response is not sufficient to explain this profound change in outcome. Using adoptive transfer of $CD8^+$ T cells, this study demonstrated unequivocally that T-cell expression of MyD88 is critical for a normal T-cell response to LCMV. In addition, it was found that expression of MyD88 is superfluous during early activation and proliferation of the antigen-activated $CD8^+$ T cells, but plays a critical role in the sustained expansion of the antigen-specific $CD8^+$ T-cell population during the primary T-cell response. Interestingly, a critical role for MyD88 was evident only under conditions of systemic infection with virus capable of causing prolonged infection, suggesting that MyD88 expression may function as an internal regulator of the threshold for antigen-driven, exhaustive differentiation.

INTRODUCTION

Myeloid differentiation factor 88 (MyD88) was originally isolated as a myeloid differentiation factor (Lord *et al.*, 1990). However, nowadays it is primarily known for its function as a key adaptor molecule required for signalling through most Toll-like receptors (TLRs) (Akira & Takeda, 2004; Medzhitov *et al.*, 1998). In this context, MyD88 serves to translate the recognition of pathogen-associated molecular patterns on the cell surface or in endosomal compartments into activation of the innate immune system and to create the conditions for optimal activation of adaptive immunity (Iwasaki & Medzhitov, 2004; Thoma-Uszynski *et al.*, 2001). Thus, TLR-mediated activation of critical subsets of antigen-presenting cells may be pivotal for the optimal induction of $CD8^+$ T-cell responses to some, but not all, viruses (Lopez *et al.*, 2006). However, MyD88 is also involved in signal transduction from members of the interleukin (IL)-1 receptor (IL-1R) family, and therefore impaired T-cell immunity in MyD88-deficient ($MyD88^{-/-}$) mice may also reflect the critical involvement of cytokines that serve as agonists for the receptors belonging to this family, e.g. IL-1 and IL-18 (Adachi *et al.*, 1998). Finally, TLRs may also be expressed by the T cells themselves (Cottalorda *et al.*, 2006; Gelman *et al.*, 2004), and recent results suggest that MyD88 may play an important role as a T-cell intrinsic differentiation factor. Thus, $MyD88^{-/-}$ mice infected with lymphocytic choriomeningitis virus (LCMV) generate a markedly impaired antiviral $CD8^+$ T-cell response. This does not, however, seem to reflect an impaired innate immune response, as T-cell receptor (TCR) transgenic (Tg), LCMV-specific, MyD88-sufficient T cells are fully activated in a MyD88-deficient environment as opposed to the endogenous $MyD88^{-/-}$ T cells present in the same environment (Zhou *et al.*, 2005). Nevertheless, the precise role of MyD88 in the generation of antiviral T-cell responses is far from fully resolved, and for this reason we decided to investigate whether T-cell intrinsic expression of MyD88 represents a pivotal requirement for the development of a normal antiviral $CD8^+$ T-cell response.

Our initial analysis revealed that the importance of MyD88 expression varied with the infection studied, and that primary intravenous (i.v.) infection with LCMV seemed to represent a uniquely sensitive case. Using adoptive transfer of polyclonal $CD8^+$ T cells, we could demonstrate unequivocally that T-cell intrinsic expression of MyD88 is essential during the primary LCMV-specific $CD8^+$ T-cell response. Therefore, in order to define better the stage at which T-cell expression of MyD88 was essential in LCMV-infected mice, TCR Tg mice deficient in MyD88 expression were generated, and the capacity of their $CD8^+$ T cells to respond to the cognate antigen in an otherwise MyD88-sufficient environment was studied *in vivo*. In this way, we showed that expression of MyD88 is superfluous during

early activation and expansion of antigen-activated T cells, but plays a critical role in the sustained accumulation of the differentiated cells during the primary $CD8^+$ T-cell response to LCMV.

METHODS

Mice. Wild-type C57BL/6 (WT) mice were purchased from Taconic M&B. IL-1R1$^{-/-}$, IL-18R1$^{-/-}$ and Thy1.1 mice, all on a C57BL/6 background, were purchased from The Jackson Laboratory. MyD88$^{-/-}$ mice were the progeny of breeder pairs obtained from S. Akira, Osaka University, Japan (Adachi *et al.*, 1998). TCR318 Tg mice, expressing a TCR for the LCMV glycoprotein epitope aa 33–41 ($GP_{33–41}$) on about 60 % of their $CD8^+$ T cells were bred locally from breeder pairs kindly provided by R. Zinkernagel, Universitätsspital, Zürich, Switzerland. MyD88$^{-/-}$ and TCR318 Tg mice were intercrossed to produce MyD88$^{-/-}$ TCR318 Tg mice. The genotype of these mice was determined by PCR (for the *MyD88* gene) and by quantitative PCR (for the TCR318 transgene) using DNA obtained from the tail. B6.SJL mice (B6.SJL-*Ptprc*^{a}/BoAiTac) carrying the CD45.1 allele were bred locally from a breeder pair obtained from The Jackson Laboratory. TLR2-deficient mice were a kind gift from S. Paludan, University of Aarhus, Denmark. Mice from outside sources were always allowed to rest for at least 1 week before being used in experiments, at which time the animals usually were 7–12 weeks old. All mice were housed under controlled (specific-pathogen-free) conditions as validated by testing of sentinels for unwanted infections according to the Federation of European Laboratory Animal Science Association standards; no such infections were detected. Animal experiments were conducted in accordance with national guidelines.

Virus. The viscerotropic LCMV Traub strain was used in most experiments. Mice to be infected received 200 p.f.u. virus in an i.v. injection of 0.3 ml; inoculation by this route results in non-lethal, immunizing infection (Kristensen *et al.*, 2002). In a few experiments, LCMV Armstrong strain (clone 53b) was used at a dose of 10^4 p.f.u. in an i.v. injection of 0.3 ml (Kristensen *et al.*, 2002). Vesicular stomatitis virus (VSV) Indiana strain was used at an i.v. dose of 10^6 p.f.u. This dose is non-lethal in immunocompetent mice and induces a distinct $CD8^+$ T-cell response (Andreasen *et al.*, 2000; Thomsen *et al.*, 1997). Replication-deficient adenovirus encoding $GP_{33–41}$ linked to β2-microglublin was produced, stored and quantified as described recently (Holst *et al.*, 2007). Mice to be vaccinated were anaesthetized and injected with 2×10^7 HEK293-infectious units in the right hind footpad.

Virus titrations. Lung virus titres were determined by an immune focus assay in MC57G cells. Lungs were first gently homogenized in PBS containing 1 % fetal calf serum (FCS) to yield a 10 % (v/w) organ suspension. Organ suspensions were clarified by centrifugation, and serial 10-fold dilutions of the supernatants were prepared in PBS with 1 % FCS. A sample of each dilution (0.2 ml) was then transferred in duplicate into flat-bottomed, 24-well plates, and MC57G cells were added in minimal essential medium (MEM). Plates were incubated for 4–6 h at 37 °C in 5 % CO_2, to allow cells to adhere. Subsequently, 0.3 ml of a 1 : 1 mixture of 2 % methylcellulose in double-distilled water and double-strength MEM with 10 % FCS, antibiotics and glutamine was added. After 48 h, cell monolayers were fixed with 4 % formaldehyde in PBS for 20–30 min at 20 °C and permeabilized in 0.5 % Triton X-100 in Hanks' balanced salt solution for 20 min. The following day, monolayers were labelled with a rat anti-LCMV monoclonal antibody (mAb) (VL-4; kindly provided by R. Zinkernagel, Universitätsspital, Zürich, Schwitzerland) for 60–90 min, washed intensively, incubated with peroxidase-labelled goat anti-rat antibody for 60–90 min and washed again. *O*-Phenylenediamine (substrate) was added for 10–30 min and the reaction was subsequently terminated by washing with water. The numbers of p.f.u. were counted, and organ virus titres were expressed as p.f.u. (g tissue)$^{-1}$ (Battegay *et al.*, 1991).

Labelling with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) and adoptive transfer experiments. Spleen cells from TCR318 Tg mice or MyD88$^{-/-}$ TCR318 Tg mice were adjusted to 1×10^7 cells ml^{-1} and mixed with CFSE to a final concentration of 1 μM. After incubation for 10 min at 37 °C, 0.1 vols FCS was added and the cells were immediately washed three times with RPMI 1640 with 10 % FCS. The cells were finally resuspended in PBS and 2×10^6 or 2×10^7 CFSE-labelled cells were adoptively transferred into B6.SJL recipients. For adoptive transfer of polyclonal populations, 2×10^7 adherent-depleted spleen cells were transplanted into the recipients.

Flow cytometric analysis. All antibodies for flow cytometry were purchased from PharMingen as rat anti-mouse mAbs. H-2D^{b}/$GP_{33–41}$ and H-2D^{b}/$NP_{396–404}$ dextramers were kindly provided by Dako.

Cells (2×10^6) were incubated with dextramers for 30 min at 4 °C in FACS medium I (PBS containing 10 % rat serum, 1 % BSA and 0.1 % NaN_3), at which time mAbs for surface labelling were added and the cells were incubated for a further 30 min. After washing twice, the cells were fixed with 1 % paraformaldehyde. To detect intracellular cytokines, splenocytes were cultured at 37 °C in a 96-well, round-bottomed microtitre plates at a concentration of 2×10^6 cells per well in a volume of 0.2 ml complete RPMI supplemented with murine recombinant IL-2 (50 U ml^{-1}), 3 μM monensin and peptide. The peptides were used at a concentration of 0.1 μg ml^{-1} [LCMV $GP_{33–41}$ and nucleoprotein $(NP)_{396–404}$] or 1 μg ml^{-1} [LCMV $GP_{61–80}$ and VSV $NP_{52–59}$]. After 5 h of culture, the cells were washed once in FACS medium II (PBS containing 1 % BSA, 0.1 % NaN_3 and 3 μM monensin) and subsequently incubated with the relevant surface antibodies in the dark for 20 min at 4 °C. The cells were washed twice in PBS plus 3 μM monensin, resuspended in 100 μl PBS, and 100 μl 2 % paraformaldehyde in PBS was added. After 30 min of incubation in the dark at 4 °C, the cells were washed in FACS medium II and resuspended in PBS with 0.5 % saponin. Following 10 min of incubation in the dark at 20 °C, the cells were pelleted and resuspended in PBS with 0.5 % saponin and the relevant antibodies. After incubation for 20 min at 4 °C, cells were washed twice in saponin and resuspended in FACS medium II. Samples were acquired on a FACSCalibur (Becton Dickinson), and at least 10^4 mononuclear cells were gated using a combination of forward angle and side scatter to exclude dead cells and debris. Data were analysed using CellQuest software.

RESULTS

Impaired T-cell responsiveness and uncontrolled virus spread during LCMV infection in MyD88-deficient mice

To determine the importance of MyD88 in the induction of the T-cell response to LCMV, MyD88$^{-/-}$ mice and matched WT mice were infected with a moderate dose of viscerotropic LCMV Traub strain, and the virus-specific T-cell response was measured at 6, 8 and 30 days post-infection (p.i.).

An early and sustained collapse of the LCMV-specific T-cell response was observed in MyD88$^{-/-}$ mice. Thus, the numbers of $GP_{33–41}$-specific $CD8^+$ T cells were signific-

antly lower in MyD88$^{-/-}$ mice compared with WT mice when measured by staining with MHC dextramers (H-2D^b/GP$_{33-41}$) (Fig. 1a). Moreover, the few GP$_{33-41}$-specific T cells remaining in MyD88$^{-/-}$ mice failed to produce gamma interferon (IFN-γ) following stimulation with GP$_{33-41}$ peptide *in vitro* (Fig. 1b). A markedly reduced response was also observed for NP$_{396-404}$-specific CD8$^+$ T cells and GP$_{61-80}$-specific CD4$^+$ T cells (Fig. 1b). The impaired T-cell response in MyD88$^{-/-}$ mice was evident by 6 days p.i. and became even more pronounced with time, eventually resulting in 2–3 logs fewer virus-specific T cells in MyD88$^{-/-}$ mice compared with matched WT mice.

Reflecting the absence of a functional virus-specific T-cell response, uncontrolled virus growth was observed in the organs of LCMV-infected MyD88$^{-/-}$ mice, whereas WT mice rapidly controlled the infection (Fig. 1c).

Compared with the LCMV Traub strain, LCMV Armstrong strain is almost incapable of causing chronic infection, and in WT mice even high doses of infection are cleared much more rapidly (Fig. 2a and Kristensen *et al.*, 2002; Nansen *et al.*, 1999). Interestingly, infection of MyD88$^{-/-}$ mice with a high dose of LCMV Armstrong did not cause nearly as marked an impairment of the antiviral CD8$^+$ T-cell response as infection with LCMV Traub. Thus, some IFN-γ-producing virus-specific T cells could be detected in LCMV Armstrong-infected, MyD88$^{-/-}$ mice at the peak of the response, 8 days p.i. (Fig. 2b). However, total numbers of GP$_{33-41}$- and NP$_{396-404}$-specific T cells were still decreased by 1–1.5 logs and – especially for NP$_{396-404}$-specific T cells – the ability of the individual T cell to produce IFN-γ (measured as mean fluorescence intensity) was significantly impaired (Fig. 2b, c). This discrepancy in T-cell impairment between LCMV Traub- and LCMV Armstrong-infected MyD88$^{-/-}$ mice may suggest that the need for MyD88 might vary with the capacity of the virus to maintain a high viral load.

LCMV is a viscerotropic, rapidly replicating virus that induces a massive virus-specific CD8$^+$ T-cell response. The profound activation of innate defence mechanisms, leading to a potent co-stimulatory environment (Thompson *et al.*, 2006), in combination with a high viral load, might render LCMV-specific CD8$^+$ T cells particularly susceptible to exhaustive differentiation (Wherry & Ahmed, 2004) and consequently more dependent on MyD88. To test this assumption, we investigated whether MyD88 played a similar role in the induction of a virus-specific CD8$^+$ T-cell response in mice infected with VSV. Following infection with this virus, activation of the innate immune system is less pronounced, viral replication is limited and, consequently, the antigenic load is low and transient (Christensen *et al.*, 2004; Thompson *et al.*, 2006; Thomsen *et al.*, 1997). VSV-infected MyD88$^{-/-}$ mice were analysed with respect to the number of virus-specific CD8$^+$ T cells present in the acute and late phases of infection (days 7 and 28 p.i., respectively).

VSV-infected MyD88$^{-/-}$ mice showed an essentially unimpaired CD8$^+$ T-cell response in the acute phase of

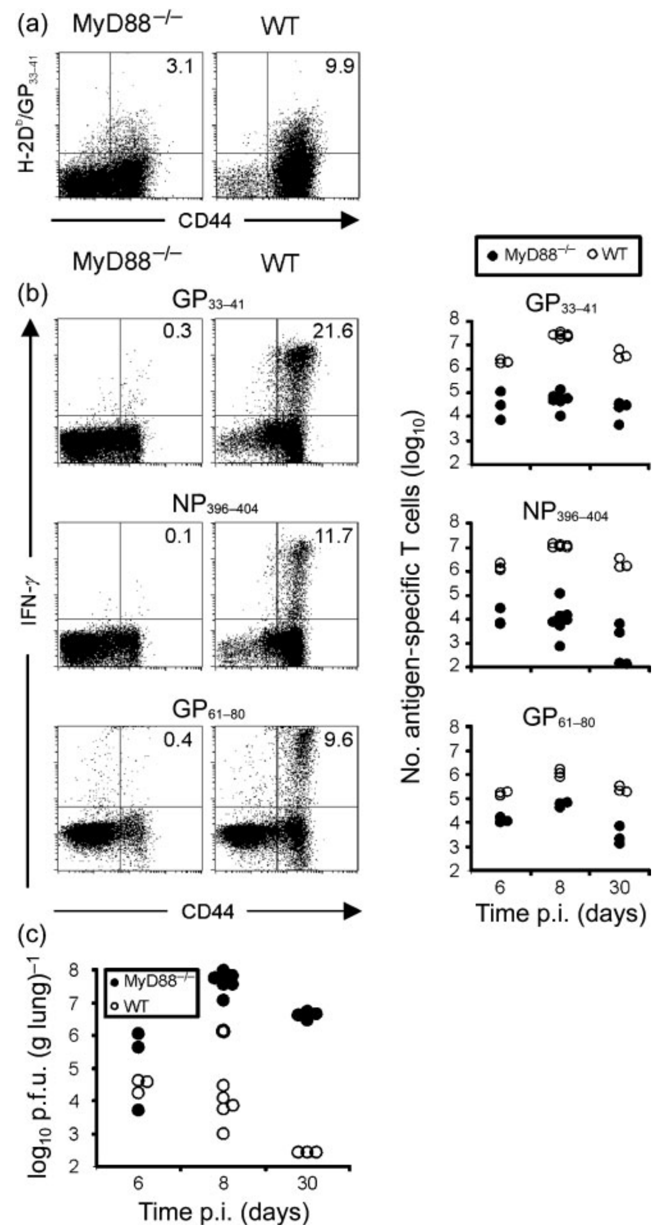

Fig. 1. Impaired T-cell responsiveness and uncontrolled virus spread during LCMV infection in MyD88-deficient mice. MyD88$^{-/-}$ and WT mice were infected with LCMV Traub. (a) Splenocytes harvested on day 8 p.i. were stained with H-2D^b/GP$_{33-41}$ dextramers and CD8 and CD44 antibodies. Representative FACS plots of gated CD8$^+$ T cells are shown. (b) Kinetics of the virus-specific T-cell response as measured by intracellular staining for IFN-γ following stimulation with viral peptides (GP$_{33-41}$, NP$_{396-404}$ or GP$_{61-80}$). Representative plots from day 8 p.i. are shown on the left and the total numbers of cytokine-producing T cells with an activated phenotype are shown on the right. Points represent individual mice. (c) Lung virus titres from the same mice as in (b).

infection compared with WT mice (Fig. 2d), indicating that MyD88 is not essential for the activation and survival of VSV-specific CD8$^+$ T cells. On day 28 p.i., three out of

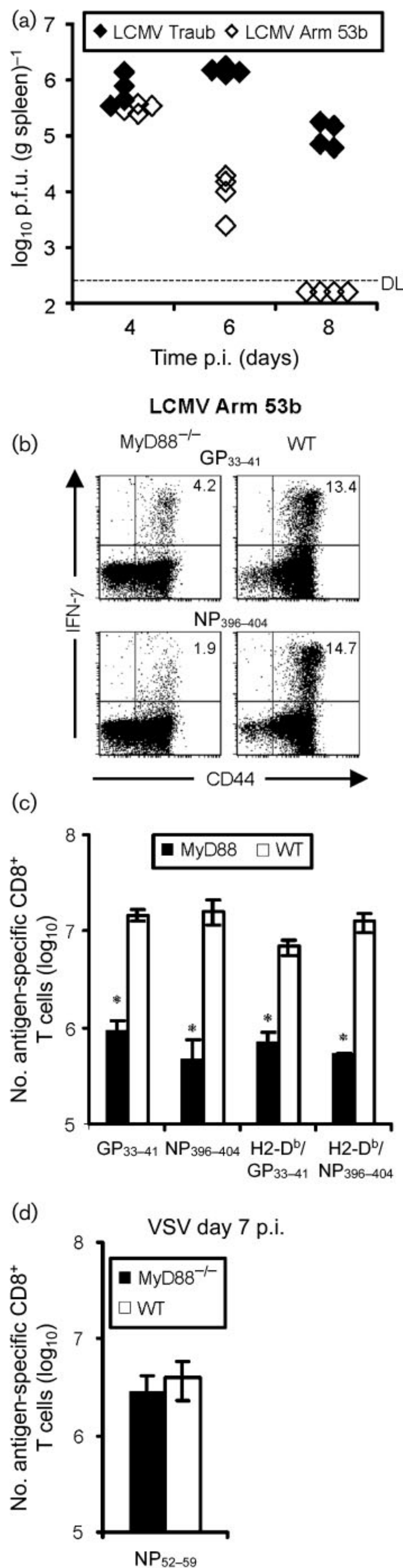

Fig. 2. The requirement for MyD88 varies with the virus used for challenge. (a) C57BL/6 mice were infected with 200 p.f.u. LCMV Traub or 10^4 p.f.u. LCMV Armstrong clone 53b (Arm 53b), and spleen virus titres were determined at 4, 6 and 8 days p.i. Points represent individual mice. (b, c) MyD88$^{-/-}$ and WT mice were infected with LCMV Armstrong clone 53b. On day 8 p.i., splenocytes were either incubated with viral peptide (GP_{33-41} or $NP_{396-404}$) and analysed for IFN-γ production by intracellular staining or surface stained with LCMV-specific MHC class I dextramers (H-2D^b/GP_{33-41} or H-2D^b/$NP_{396-404}$). The results are shown as representative plots of gated CD8$^+$ T cells following intracellular staining for IFN-γ (b) and total numbers of cytokine-producing- or dextramer-binding, CD44^{high} CD8$^+$ T cells specific for GP_{33-41} or $NP_{396-404}$ (means ± SD for three mice) (c). *, $P<0.05$ relative to matched controls. (d) MyD88$^{-/-}$ and WT mice were infected with VSV. On day 7 p.i., splenocytes were incubated with viral peptide (NP_{52-59}) for 5 h and analysed for IFN-γ production by intracellular staining. The results are shown as total numbers of IFN-γ-producing CD8$^+$ T cells with an activated phenotype (means ± SD for three mice).

four MyD88$^{-/-}$ mice had succumbed to VSV infection, which is in agreement with previous findings (Zhou *et al.*, 2007), suggesting a critical role for MyD88 in the humoral immune response to this virus. The one surviving MyD88$^{-/-}$ mouse had an antiviral CD8$^+$ T-cell response comparable to its WT counterparts (data not shown). These findings are consistent with the hypothesis that the requirement for MyD88 in generation and/or maintenance of antiviral T-cell responses applies primarily to virus infections associated with a prolonged and high viral load.

MyD88 is not required during the response to LCMV in antigen-experienced mice

To study the requirement for MyD88 during a recall response to LCMV, we generated LCMV-specific memory T cells in MyD88$^{-/-}$ mice by immunization with replication-deficient adenovirus encoding the GP_{33-41} epitope linked to human β2-microglobulin (Ad–GP33). We have shown previously that immunization of WT mice with this construct efficiently induces a GP_{33-41}-specific T-cell response that peaks after 2–3 weeks (Holst *et al.*, 2007). MyD88$^{-/-}$ and WT mice were immunized with 2×10^7 infectious units Ad–GP33, and the GP_{33-41}-specific T-cell response was analysed at 14 and 80 days post-vaccination. Following priming in this manner, GP_{33-41}-specific T cells were induced and maintained at significant numbers for at least 80 days post-vaccination in the absence of MyD88 expression (Fig. 3). Importantly, the vaccination-induced GP_{33-41}-specific T cells in MyD88$^{-/-}$ mice were functional and could expand during subsequent challenge with the same dose of virus (200 p.f.u. LCMV Traub) that completely inhibited the response in naïve MyD88$^{-/-}$ mice (Fig. 3, day 80 + 5). Thus, whereas MyD88 is crucial for the induction of a primary CD8$^+$ T-cell response to LCMV, MyD88 expression seems to be redundant during

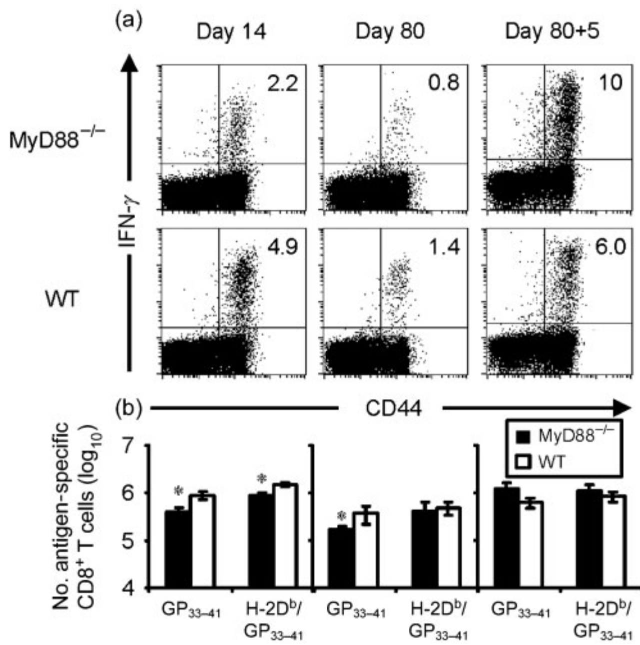

Fig. 3. MyD88 is not required in antigen-experienced mice. MyD88$^{-/-}$ and WT mice were immunized in the footpad with Ad–GP$_{33-41}$, and the GP$_{33-41}$-specific T-cell response was analysed 14 or 80 days post-immunization. On day 80, some mice were infected with LCMV Traub, and the GP$_{33-41}$-specific recall response was analysed 5 days later (day 80+5). Splenocytes were either incubated with GP$_{33-41}$ peptide for 5 h and analysed for IFN-γ production by intracellular staining or surface stained with H-2D^b/GP$_{33-41}$ dextramers. (a) Representative plots of gated CD8$^+$ T cells following intracellular staining for IFN-γ. (b) Total numbers of cytokine-producing- or dextramer-binding CD8$^+$ T cells specific for GP$_{33-41}$ or NP$_{396-404}$ (means ± SD for four to five mice). *, $P<0.05$ relative to matched controls.

the secondary response where virus replication is rapidly controlled, as shown previously by Holst *et al.* (2007).

MyD88 does not mediate its role through IL-1R, IL-18R or TLR2

MyD88 was first characterized functionally as an adaptor molecule required for the IL-1 cytokine family (IL-1/IL-18)-induced signalling pathway (Adachi *et al.*, 1998). To investigate whether MyD88 mediates its critical function through this pathway during primary LCMV infection, IL-1R1- and IL-18R1-deficient mice were infected with LCMV Traub, and the antiviral CD8$^+$ T-cell response was measured 8 days later by intracellular staining for IFN-γ. The number (Fig. 4a) and quality (not shown) of GP$_{33-41}$- and NP$_{396-404}$-specific CD8$^+$ T cells was similar in IL-1R$^{-/-}$, IL-18R$^{-/-}$ and WT mice, indicating that the critical role of MyD88 in the LCMV-specific T-cell response is not mediated through the IL-1/IL-18 signalling pathway. In agreement with previous findings (Zhou *et al.*, 2005), the absence of TLR2 also had no effect on the LCMV-specific CD8$^+$ T-cell response (Fig. 4b).

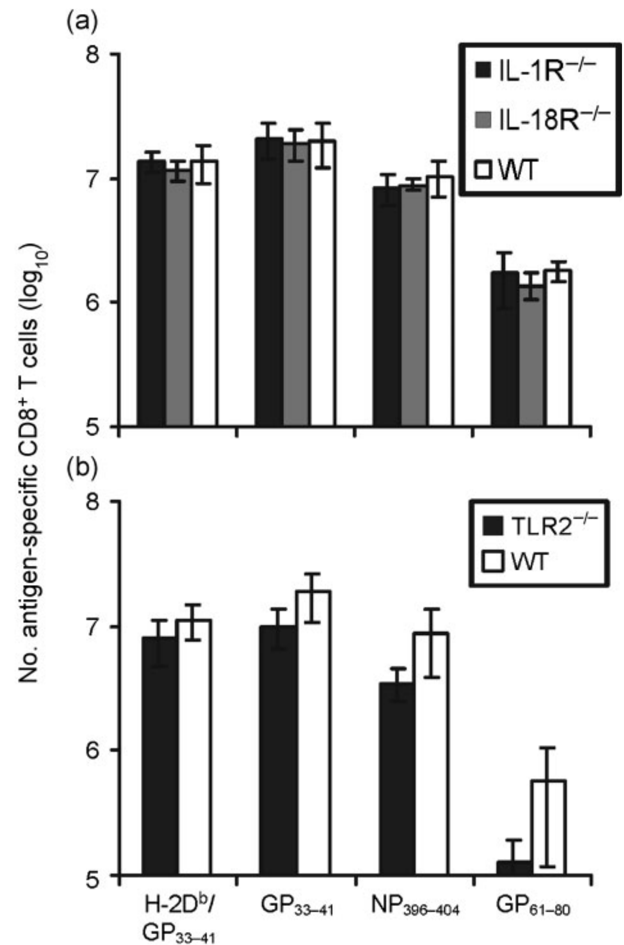

Fig. 4. The critical role of MyD88 in the LCMV-specific T-cell response is not mediated through the IL-1/IL-18 or TLR2 signalling pathway. IL-1R1$^{-/-}$, IL-18R1$^{-/-}$, and WT mice (a) or TLR2$^{-/-}$ and WT mice (b) were infected with LCMV Traub, and splenocytes were analysed 8 days later for LCMV-specific CD8$^+$ T cells as described in Figs 1–3. The results are shown as the total numbers of cytokine-producing or dextramer-binding CD8$^+$ T cells specific for GP$_{33-41}$ or NP$_{396-404}$ (means ± SD for five to six mice).

Polyclonal MyD88$^{-/-}$ CD8$^+$ T cells expand and differentiate poorly in a WT environment

MyD88 has been described primarily as an adaptor molecule involved in the TLR signalling cascade in antigen-presenting cells. However, Zhou *et al.* (2005) recently provided preliminary evidence that MyD88 might also act directly at the T-cell level during LCMV infection. This was demonstrated by the use of TCR Tg, MyD88-sufficient P14 cells specific for the immunodominant LCMV epitope GP$_{33-41}$, adoptively transferred into MyD88$^{-/-}$ mice (Zhou *et al.*, 2005). In this set-up, the altered precursor frequency in MyD88$^{-/-}$ mice could,

however, have influenced the result. To study more appropriately whether MyD88 expression in T cells themselves plays a role, we compared the expansion and differentiation of non-transgenic, naïve MyD88$^{-/-}$ and MyD88$^{+/+}$ T cells in a MyD88-sufficient environment. Adherent cell-depleted spleen cells from MyD88$^{-/-}$ mice or WT (CD45.2) mice were adoptively transferred into MyD88-competent B6.SJL (CD45.1) mice. The following day, recipients were infected with LCMV Traub, and the LCMV-specific CD8^{+} T-cell response was analysed 8 days later by intracellular staining for IFN-γ. Donor cells were distinguished from recipient cells by the CD45.2 marker. Despite the MyD88-intact environment in recipient mice, fewer donor CD8^{+} T cells were recovered from recipients given MyD88$^{-/-}$ cells, and few if any GP$_{33-41}$- and NP$_{396-404}$-specific effector T cells from MyD88-deficient mice could be detected in recipients' spleens (Fig. 5). In contrast, a distinct population of donor-derived, IFN-γ-producing GP$_{33-41}$- and NP$_{396-404}$-specific T cells was present in recipients given similar numbers of WT donor cells. Similar results were obtained when donor cells from MyD88$^{-/-}$ mice (CD45.2^{+}, Thy1.2) and WT B6.SJL mice (CD45.1^{+}, Thy1.2) were co-transferred into the same Thy1.1 recipient mice (data not shown). These findings clearly confirm and extend the suggestion that part of the critical role for MyD88 in the induction of a functional T-cell response during primary LCMV infection requires the expression of MyD88 in the T cells themselves.

MyD88 is important for the continued expansion rather than the activation of virus-specific CD8^{+} T cells during LCMV infection

In order to study at which stage(s) during the primary LCMV-specific T-cell response MyD88 exerts its critical function in the T cells, we generated MyD88$^{-/-}$ mice transgenic for the GP$_{33-41}$ immunodominant epitope (MyD88$^{-/-}$ TCR318 Tg mice). In these mice, 50–60 % of the MyD88-deficient CD8^{+} T cells are specific for the GP$_{33-41}$ epitope. The *in vivo* proliferative capacity of LCMV-specific MyD88$^{-/-}$ T cells was analysed first. Spleen cells from MyD88$^{-/-}$ or MyD88$^{+/+}$ TCR318 Tg (CD45.2^{+}) mice were labelled with CFSE, and 2×10^{7} labelled spleen cells were then adoptively transferred into B6.SJL (CD45.1^{+}) mice. Recipient mice were infected with LCMV Traub the following day, and the proliferation of donor cells was measured as CFSE dilution using flow cytometry on days 3 and 4 p.i. (Fig. 6a, b). Surprisingly, MyD88$^{-/-}$ TCR318 donor T cells proliferated as well as their WT counterparts, and equal numbers of donor CD8^{+} T cells were recovered from recipients' spleens at 3 and 4 days p.i. In fact, MyD88$^{-/-}$ TCR318 Tg cells tended to proliferate slightly faster than their WT counterparts, indicating that MyD88 is not required for the initial activation of virus-specific T cells during LCMV infection.

To ascertain that this finding did not simply reflect an unphysiologically high frequency of LCMV-specific precursors, we reduced the number of donor cells by a factor of 10 and again investigated the fate of MyD88$^{-/-}$ donor T cells during subsequent LCMV infection. Spleen cells (2×10^{6}) from MyD88$^{-/-}$ or MyD88$^{+/+}$ TCR318 Tg mice were transferred to B6.SJL recipients, and the numbers of donor CD8^{+} T cells were measured at 4, 6, 8 and 12 days after LCMV infection (Fig. 6c). Again, on day 4 p.i., similar if not higher numbers of donor CD8^{+} T cells could be recovered from the spleens of recipients receiving MyD88$^{-/-}$ cells compared with MyD88$^{+/+}$ cells. However, we did not see any expansion of the MyD88$^{-/-}$ donor population after day 6, and, as a consequence, the virus-specific CD8^{+} T-cell population began to contract between

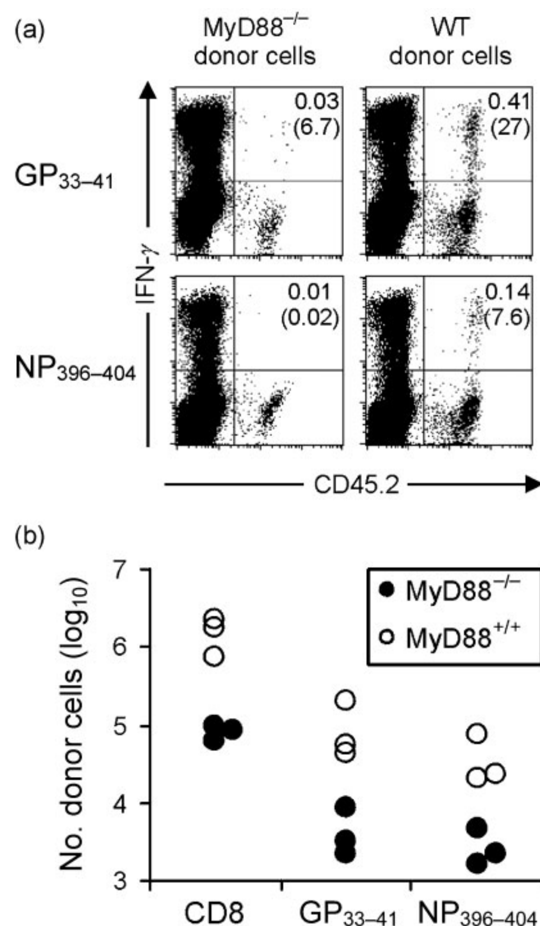

Fig. 5. Polyclonal MyD88$^{-/-}$ CD8^{+} T cells expand and differentiate poorly in a WT environment. Spleen cells (2×10^{7}) from MyD88$^{-/-}$ or WT mice were adoptively transferred into B6.SJL (CD45.1^{+}) mice. The day after the cell transfer, all mice were infected with LCMV Traub. Eight days later, splenocytes were incubated with GP$_{33-41}$ or NP$_{396-404}$ peptide for 5 h and analysed for IFN-γ production by intracellular staining. (a) Representative plots of gated CD8^{+} T cells are shown; numbers in parentheses refer to the percentage of IFN-γ producers from the donor (CD45.2^{+}) CD8^{+} cells. (b) Total numbers of donor cells. Points represent individual mice.

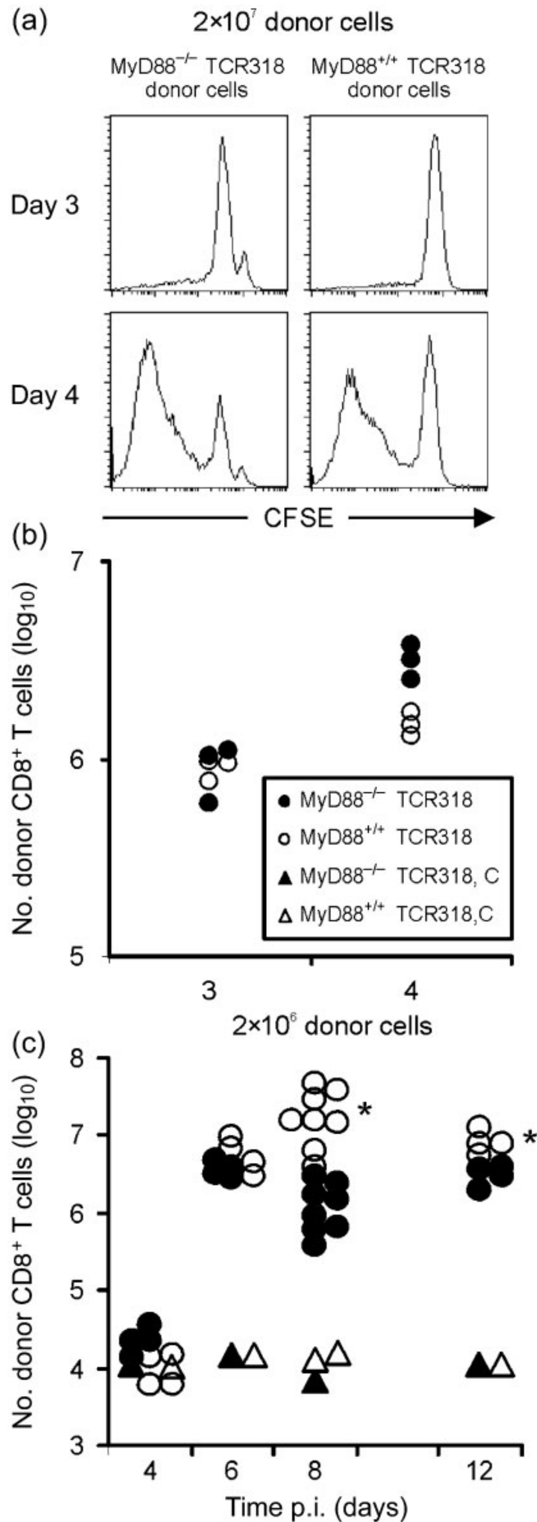

Fig. 6. MyD88$^{-/-}$ T cells initially proliferate normally, but are unable to undergo sustained expansion during acute LCMV infection. (a, b) Spleens from MyD88$^{-/-}$ or MyD88$^{+/+}$ TCR318 Tg mice were labelled with CFSE, and 2×10^7 cells were transferred into B6.SJL (CD45.1$^+$) recipients. The day after cell transfer, mice were infected with LCMV Traub. (a) Representative plots of gated donor CD8$^+$ T cells (CD45.2$^+$) at days 3 and 4 p.i. (b) Total numbers of donor CD8$^+$ T cells at 3 and 4 days p.i. Points represent individual mice. (c) Splenocytes (2×10^6) from MyD88$^{-/-}$ or MyD88$^{+/+}$ TCR318 Tg mice were adoptively transferred into B6.SJL recipients. The day after cell transfer, mice were infected with LCMV Traub. Uninfected recipients served as controls (TCR318, C). The results are shown as total numbers of donor CD8$^+$ T cells at 4, 6, 8 and 12 days p.i. Points represent individual mice; *, $P<0.05$ relative to MyD88-deficient cells.

days 6 and 8 p.i. This was in contrast to the case for MyD88$^{+/+}$ T cells, which continued to expand during this interval creating a difference of ~1 log in donor cell numbers on day 8 p.i. Between days 8 and 12 p.i., the numbers of MyD88$^{-/-}$ donor cells stabilized whilst the normal contraction of MyD88$^{+/+}$ donor cells was now observed. These findings suggest that MyD88 expression in the activated CD8$^+$ T cells is required for prolonged expansion rather than for initial activation of the virus-specific T cells during acute LCMV infection.

DISCUSSION

In the present study, we have confirmed and extended earlier findings pointing to an important role for T-cell intrinsic expression of MyD88 in the CD8$^+$ T-cell response to systemic infection with LCMV. Thus, whilst an earlier study by Zhou *et al.* (2005) only provided suggestive evidence for a T-cell intrinsic role of MyD88 expression, the present results directly demonstrated that MyD88-deficient CD8$^+$ T cells are impaired in their capacity to respond to antigen activation, even in a MyD88-sufficient environment. We also clearly demonstrated that this is true not only for TCR Tg cells, but also for a normal polyclonal CD8$^+$ T-cell population.

Using MyD88$^{-/-}$ TCR Tg CD8$^+$ T cells, we were also able to dissect more precisely the stage at which MyD88 expression is required. Thus, based on analysis of CFSE dilution and donor CD8$^+$ T-cell numbers, it was found that initial activation and expansion of the LCMV-specific CD8$^+$ T cells did not require T-cell intrinsic expression of MyD88. However, subsequent accumulation of MyD88$^{-/-}$ CD8$^+$ T cells in the spleen was significantly reduced and, as a result, the antigen-specific CD8$^+$ T-cell population began to contract earlier than did matched MyD88$^{+/+}$ T cells.

Our results also revealed that the requirement for expression of MyD88 is not absolute, but varies with the viral infection studied. A pertinent question, therefore, is why T-cell expression of MyD88 is not universally required during the antiviral CD8$^+$ T-cell response. Our results do not provide a definite answer to this question. However, in this context, it is of interest to note that the behaviour of MyD88$^{-/-}$ CD8$^+$ T cells is very similar to that of WT CD8$^+$ T cells under conditions of high-dose infection (~10^6 p.f.u., i.v.) with invasive strains of LCMV (e.g. clone 13) (Kristensen *et al.*, 2002; Wherry & Ahmed, 2004).

Moreover, using a graded spectrum of conditions for $CD8^+$ T-cell activation (LCMV Traub, LCMV Armstrong, VSV, Ad–GP33), results were obtained that could suggest that a prolonged systemic viral load might be essential in revealing the importance of T-cell-expressed MyD88. Such an association would also explain why the same viral challenge in naïve and in antigen-experienced mice may lead to quite different conclusions regarding the importance of MyD88. Thus, in naïve mice, inoculation of LCMV Traub rapidly leads to a high viral load in several internal organs, whereas in vaccinated mice challenged with the same virus dose, virus replication is rapidly controlled (Holst *et al.*, 2007) and a 'suppressive' environment is not likely to be established.

If our interpretation of the experimental results is correct, T-cell intrinsic expression of MyD88 is likely to be important only in connection with a limited number of viral infections, namely those that may result in chronic systemic infection. In humans, this could be human immunodeficiency virus, hepatitis B and C viruses or perhaps human cytomegalovirus. In most human viral infections, either the respiratory tract or the gastrointestinal tract is the primary target and little viral invasion is observed. Therefore, such infections may not reveal a critical role for MyD88, at least not in the T cells themselves. However, the immune response to the latter type of infection may be more susceptible to the absence of MyD88 expression in dendritic cells, as superficial/mucosal infections are likely to represent less efficient inducers of essential co-stimulatory signals.

Our study did not reveal which upstream receptor(s) are using MyD88 as an adaptor in the $CD8^+$ T cells. Neither IL-1R- or IL-18R-deficient mice expressed the same immunodeficient phenotype as similarly infected $MyD88^{-/-}$ mice, thus ruling out the most obvious candidates. It has been found previously that TLR2 and TLR9 ligation augment the proliferation of murine T cells *in vitro*, and TLR2 may function as a co-stimulatory co-receptor on activated T cells (Cottalorda *et al.*, 2006; Gelman *et al.*, 2004). However, confirming earlier results (Zhou *et al.*, 2005), TLR2 mice generated an essentially normal LCMV-specific $CD8^+$ T-cell response, and there is no reason why TLR9 should play a major role during infection with an RNA virus. Moreover, a recent study revealed that TLR9-deficient mice generated an almost normal $CD8^+$ T-cell response to LCMV (Jung *et al.*, 2008). One explanation for these negative results could be that several receptors are involved and that analysis of mouse strains with individually targeted genes will not reveal the critical receptors. Alternatively, MyD88 may act in $CD8^+$ T cells as an adaptor for molecules other than those classically defined. Interestingly, the behaviour of MyD88-deficient $CD8^+$ T cells bears a striking resemblance to that of similar type I IFN receptor-deficient cells (Aichele *et al.*, 2006; Kolumam *et al.*, 2005). Thus, it is tempting to try to infer some mechanistic association of the defects, particularly as MyD88 might be involved in the regulation of type I IFN production. However, although still controversial, serum levels of type I IFN in LCMV-infected, MyD88-deficient mice have been reported to be reduced only slightly (Zhou *et al.*, 2005). More importantly, in the adoptive transfer situation, the minority of MyD88-deficient T cells behave abnormally despite being in a WT environment. Thus, unless one assumes a direct link between the type I IFN receptor signalling pathway and MyD88, the underlying molecular events are likely to be different despite a similar behaviour of the deficient cells.

In conclusion, our results unequivocally demonstrate a critical role for T-cell intrinsic expression of MyD88, although this was revealed only under conditions of a prolonged systemic viral load. Under these circumstances, MyD88 seems to be required for the sustained expansion of the activated cells, perhaps by increasing the threshold for antigen-driven exhaustive differentiation (Wherry & Ahmed, 2004). Hence, absence of MyD88 expression in the T cells may result in premature contraction of the antiviral $CD8^+$ T-cell response. As contraction of the effector T-cell response prior to virus clearance in itself will lead to prolonged antigenic stimulation, a vicious circle may be initiated, which under certain conditions may result in a chronic viral infection. Consistent with this interpretation, we observed an almost complete exhaustion of the antiviral $CD8^+$ T cells in intact $MyD88^{-/-}$ mice, whilst a residual population of MyD88-deficient cells remained upon adoptive transfer into WT recipients, which have an additional, fully functional $CD8^+$ T-cell subset.

Note added in proof

After submission of this paper, a similar report was published by Rahmen *et al.* (2008).

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