Virus-specific MHC class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection

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A transgenic mouse expressing MHC class II-restricted TCR with specificity for a lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived T helper cell epitope was developed to study the role of LCMV-specific $CD4^+$ T cells in virus infection *in vivo*. The majority of $CD4^+$ T cells in TCR transgenic mice expressed the transgenic receptor, and LCMV glycoprotein-specific TCR transgenic $CD4^+$ T cells efficiently mediated help for the production of LCMV glycoprotein-specific isotype-switched antibodies. In contrast, LCMV glycoprotein-specific TCR transgenic mice exhibited a drastically reduced ability to provide help for the generation of antibody responses specific for the virus-internal nucleoprotein, indicating that intramolecular/intrastructural help is limited to antigens that are accessible to B cells on the viral surface. Antiviral cellular immunity was studied with noncytopathic LCMV and recombinant cytopathic vaccinia virus expressing the LCMV glycoprotein. TCR transgenic mice failed to efficiently control LCMV infection, demonstrating that functional LCMV-specific $CD4^+$ T cells – even if activated and present at extremely high frequencies – cannot directly mediate protective immunity against LCMV. Despite the fact that LCMV-primed $CD4^+$ T cells from TCR transgenic mice as well as from control mice showed low MHC class II-restricted cytotoxic activity *in vivo*, this did not correlate with protection against LCMV replication *in vivo*. In contrast, $CD4^+$ T cells from TCR-transgenic mice mediated efficient protection against infection with recombinant vaccinia virus. These results further support the need for different immune effector functions for protective immunity against different viral infections.

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1 Introduction

Infection of mice with noncytopathic lymphocytic choriomeningitis virus (LCMV) induces a vigorous cellular and humoral immune response [1]. The cellular mechanisms responsible for the control of LCMV infection have been studied in great detail. Infection of mice with a low dose of LCMV leads to the generation of a strong CTL response that eliminates LCMV within 8 days via a perforin-dependent pathway [2]. In contrast, infection of mice with high doses of LCMV has been shown to induce exhaustion of LCMV-specific CTL, ultimatively leading to a life-long virus carrier status [3]. The demonstration that LCMV-specific $CD8^+$ T cells are deleted due to T cell exhaustion or in neonatally infected LCMV carrier mice has been greatly facilitated by the use of TCR transgenic mice [4, 5]. Because no such model was available for LCMV-specific $CD4^+$ T cells, much less is known about the fate of LCMV-specific $CD4^+$ T cells in LCMV carrier mice.

Although the role of $CD8^+$ T cells in protection from LCMV infection is clearly established, the importance of $CD4^+$ T cells is less clear. While CD4-deficient mice can eliminate LCMV, it has been shown that a virus carrier status is more easily established in these mice [6]. In addition, lytic $CD4^+$ T cells specific for LCMV have been characterized *in vitro* [7], have been implied in T cell-mediated choriomeningitis *in vivo* [8] and a $CD4^+$ T cell

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Abbreviations: P13: Peptide 13 **P61:** Peptide 61 **GP:** Glycoprotein **LCMV:** Lymphocytic choriomeningitis virus **NP:** Nucleoprotein **VSV:** Vesicular stomatitis virus

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clone has been shown to protect against lethal LCMV infection [9]. Thus, $CD4^+$ T cells may prove pivotal in some circumstances. This notion is supported by the finding that the presence of $CD4^+$ T cells and B cells is crucial for long-term protective immunity against LCMV infection [10, 11]. In addition, viral clearance after infection with certain LCMV isolates, which cause prolonged infection, requires long-term CTL responsiveness, which was dependent on the presence of $CD4^+$ T cells [6, 12].

Humoral immune response against LCMV are T cell dependent and therefore require help by $CD4^+$ T cells for both the induction of IgM and isotype-switched IgG responses [13–16]. LCMV-specific humoral immune responses may be divided into two groups differing in the kinetics of their appearance: those binding but not neutralizing LCMV and those neutralizing LCMV. Whereas LCMV NP- or GP-binding isotype-switched antibody responses are readily induced at high titers upon infection and are measurable by ELISA already 8 days after infection, LCMV-neutralizing antibody responses usually appear only very late after infection (>60 days after infection) and show only moderate titers. Whether or not or to which extent a possibly inefficient $CD4^+$ T cell response to LCMV is involved in the late appearance of LCMV neutralizing antibody titers is unclear.

To study these aspects of $CD4^+$ T cell-mediated immunity against viruses, we generated MHC class II-restricted TCR transgenic mice with specificity for LCMV GP. This report describes the generation of this new T helper TCR transgenic mouse model and the evaluation of (i) the fate of $CD4^+$ T cells in virus carrier mice, (ii) the influence of abundant LCMV GP-specific T help in the induction of antibody responses to viral surface and virus-internal antigens and (iii) the ability of $CD4^+$ T cells to interfere with viral replication.

2 Results

2.1 Generation of transgenic mice expressing an MHC class II-restricted TCR specific for LCMV GP P13

A $CD4^+$ T cell clone (clone 1) specific for the LCMV GP-derived I-A^b binding epitope (P13 GP amino acids 61–80) was generated. The variable regions of the TCR expressed by clone 1 are composed of the Vα2.3 and the Vβ8.3 V elements (data not shown, EMBL nucleotide sequence database accession numbers Y11102 and Y11103). The genomic variable region genes were cloned into TCR cosmid expression vectors that are composed of genomic TCR loci sequences which were previously shown to allow efficient expression of rearranged TCR genes [17]. Co-injection of Vα2 and Vβ8.3 expression vectors into fertilized C57BL/6 eggs and backcrossing of a founder mouse to C57BL/6 mice gave rise to two TCR transgenic mouse lines (Smarta1 and Smarta2).

2.2 Transgenic TCR expression on thymocytes

To evaluate thymic positive and negative selection of the T cells expressing the I-A^b-restricted transgenic TCR exhibiting specificity for the LCMV GP-derived epitope P13, thymocytes of transgenic mice of the selecting H-2^b background or of the nonselecting H-2^d background as well as control thymocytes of negative littermates were subjected to FACS analysis. In addition, thymocytes from TCR transgenic LCMV carrier mice were assessed for negative selection. Fig. 1A presents three-color cytofluorimetric analysis of thymocytes from negative littermates (panel A) and from Smarta1 (panel B) and Smarta2 (panel C) TCR transgenic mice that were all of the positively selecting H-2^b background. No positive selection of the transgenic TCR occurred in the H-2^d genetic background (not shown). In panel D, thymocytes from TCR transgenic LCMV carrier mice are shown. Similar results were obtained for both TCR transgenic mouse lines. Thymocyte numbers of TCR transgenic mice were comparable to those of negative littermates (data not shown). As found previously for other TCR transgenic mice expressinig MHC class II-restricted TCR [17–21], Smarta1 and Smarta2 transgenic TCR transgenic mice showed a profound increase in the numbers of $CD4^+CD8^-$ thymocytes with a concomitant decrease in numbers of $CD4^-CD8^+$ thymocytes. Smarta1 and Smarta2 transgenic mice exhibited differently skewed $CD4^+$ T cell development, the CD4:CD8 ratio of single positive thymocytes was 27:1 and 6:1 for Smarta1 versus Smarta2 mice, respectively.

TCR transgenic LCMV carrier mice revealed an increased population of double negative thymocytes and virtual absence of double positive thymocytes (Fig. 1A, panel D). Emerging $CD4^+$ single positive thymocytes did not express the transgenic TCR. Thus, negative selection of the TCR transgene-expressing thymocytes occurred at the transition from the double negative to the double positive developmental stage, leading to deletion of the LCMV-specific TCR transgenic thymocytes. Thymocyte numbers of TCR transgenic LCMV carrier mice were reduced by 50–70 % as compared to control transgenic mice.

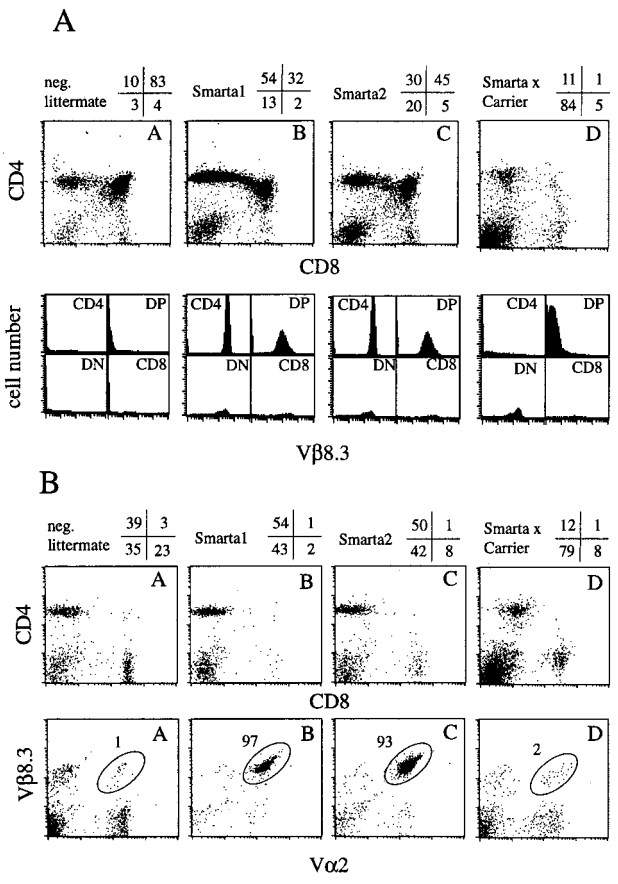

Figure 1. Cytofluorimetric analysis of thymocytes and mesenteric lymph node cells. In (A), thymocytes from negative littermates (panel A), from TCR transgenic mouse lines Smarta1 (panel B) and Smarta2 (panel C) and from TCR transgenic LCMV carrier mice (panel D) were triple stained with PE-labeled anti-CD4, FITC-labeled anti-CD8 and biotinylated anti-Vβ8.3-specific monoclonal antibody 7G8 followed by incubation with tricolor-conjugated streptavidin. Vβ8.3 staining is shown for each $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^+$ and $CD4^+CD8^-$ thymocyte population, percentages are shown above the panels. Equivalent results were obtained when analyzing expression of the transgenic Vα2 chain. Representative stainings of four experiments are shown. In (B), mesenteric lymph node cells were either double stained with phycoerythrin-labeled anti-CD4 and FITC-labeled anti-CD8 monoclonal antibodies (first row, percentages are shown above the panels) or triple stained with FITC-labeled anti-CD4, phycoerythrin-labeled anti-Vα2 and biotinylated anti-Vβ8.3 monoclonal antibodies followed by incubation with tricolor-conjugated streptavidin (second row). Live cells were gated on $CD4^+$ T cells and numbers above circles indicate the percentages of TCR transgenic T cells of the total number of $CD4^+$ T cells. Representative stainings of four experiments are shown.

2.3 Transgenic TCR expression on peripheral T cells

In order to characterize peripheral T cell populations, three-color cytofluorimetric analysis was performed using mesenteric lymph node cells from Smarta1 (Fig. 1B, panel B), Smarta2 (panel C) transgenic mice, from negative littermates (panel A) and from TCR transgenic LCMV carrier mice (panel D). Similarly as observed in thymocyte analysis, peripheral T cells from TCR transgenic Smarta1 and Smarta2 revealed a profound skewing of the CD4:CD8 ratio towards the CD4 compartment: The normal CD4:CD8 ratio of 1.8:1 in negative littermates was shifted to a CD4:CD8 ratio of 27:1 in Smarta1 and 8:1 in Smarta2 mice (Fig. 1B first row). Transgenic TCR expression on peripheral $CD4^+$ T cells is shown in the second row of Fig. 1B. More than 95 % of peripheral $CD4^+$ T cells express the transgenic TCR in the case of Smarta1 transgenics and more than 90 % in the case of Smarta2 transgenics; normal control mice exhibit only 1–2 % of $V\alpha2^+V\beta8.3^+$ peripheral T cells. In the peripheral $CD4^+$ T cell pool of TCR transgenic LCMV carrier mice, no sizable population of transgene-expressing $CD4^+$ T cells was detected, indicating again that central tolerance induction was efficient.

2.4 Functional assessment of antigen specificity of TCR transgenic $CD4^+$ T cells

To asses LCMV-specific proliferative responses, splenocytes from Smarta1 TCR transgenic animals, from negative littermates, from TCR transgenic and from nontransgenic LCMV carrier mice were stimulated with threefold serial dilutions of the indicated antigens (Fig. 2A). Transgenic T cells were specifically activated by LCMV and by the LCMV GP-derived peptide P13 but not by the irrelevant LCMV NP-derived peptide P61. $CD4^+$ T cells from naive control mice, from TCR transgenic or from nontransgenic LCMV carrier mice did not respond to LCMV-derived antigens (Fig. 2A), even in the presence of exogenously added rIL-2 (data not shown). In addition, cytokine secretion analysis revealed that transgenic $CD4^+$ T cells secreted IL-2 upon stimulation with the relevant antigens, while $CD4^+$ T cells from negative littermates or from transgenic or nontransgenic LCMV carrier mice did not secrete IL-2 after stimulation with the same antigens (Fig. 2B). Similar results were obtained for IFN-γ, whereas no IL-4 could be detected in the supernatants of activated transgenic $CD4^+$ T cells (data not shown).

Activation of TCR transgenic $CD4^+$ T cells was analyzed *in vivo* by performing adoptive transfers of TCR transgenic $CD4^+$ T cells into naive C57BL/6 recipients fol-

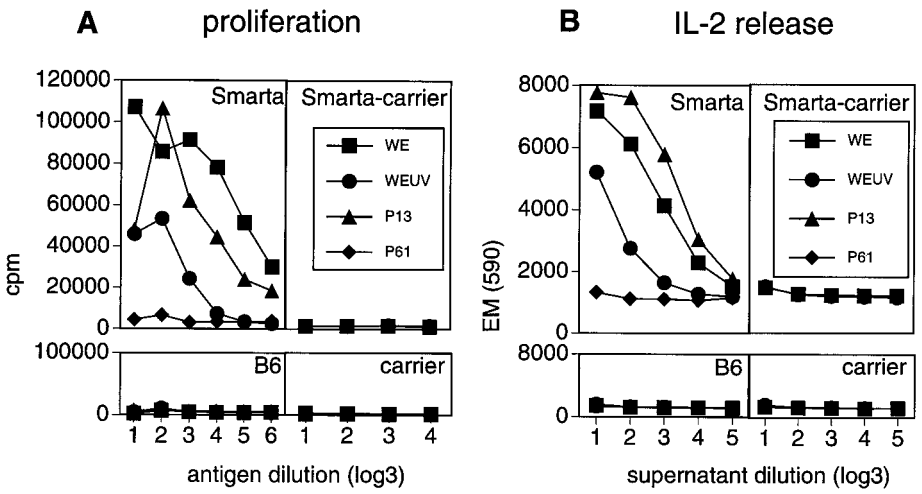

Figure 2. In vitro analysis of TCR transgenic CD4$^+$ T cells. Proliferative responses of naive TCR transgenic CD4$^+$ T cells and of CD4$^+$ T cells from TCR transgenic carrier mice is shown (A, upper panels). In addition, CD4$^+$ T cells from negative littermates or from nontransgenic carrier mice were included (A, lower panels). CD4$^+$ T cells were incubated with threefold serial dilutions of either live LCMV (squares), UV-inactivated LCMV (circles), peptide P13 (triangles) or peptide P61 (diamonds) in the presence of irradiated C57BL/6 spleen cells. T cell proliferation was determined by incorporation of [^{3}H] thymidine. One of four comparable experiments is shown. (B) Cytokine secretion of *in vitro* activated CD4$^+$ T cells. Supernatants of the proliferation assay described in (A) were collected and IL-2 contents in threefold serially diluted supernatants were determined using the IL-2 dependent cell line CTLL-2.

lowed by LCMV infection. Transferred CD4$^+$ T cells expanded in the infected recipients until they represented up to 60 % of the total CD4$^+$ PBL, demonstrating that they were activated *in vivo* and had clonally expanded (data not shown).

2.5 Antibody responses in TCR transgenic mice

To analyze the ability of TCR transgenic CD4$^+$ T cells *in vivo* to provide cognate help to B cells and for LCMV-specific antibody responses, transgenic mice and negative littermates were infected with LCMV; LCMV GP2- and LCMV NP-specific IgG antibody titers were determined by ELISA (Fig. 3). Interestingly, TCR transgenic mice almost exclusively produced LCMV GP2-binding but virtually no NP-specific IgG antibodies (Fig. 3, left panels). Mice depleted of CD8$^+$ T cells showed equivalent patterns of LCMV-specific IgG titers. In contrast, LCMV-specific IgG production was abrogated after depletion of CD4$^+$ T cells. Despite abundantly present GP-specific T help in TCR transgenic animals, they did not produce detectable levels of LCMV-neutralizing antibodies for up to 60 days after infection comparable to nontransgenic control mice (data not shown).

2.6 Absence of functional T help in LCMV carrier mice

In order to investigate LCMV-specific T cell help directly *in vivo* and independently of LCMV-specific B cells, the capacity of LCMV-specific CD4$^+$ T cells to provide cognate help to B cells specific for the hapten dinitrophenol (DNP) was assessed. DNP was chosen as B cell epitope since it is known that DNP-specific B cells exhibit a high precursor frequency [22] as compared to virus-specific B cells [23, 24]. T cell help is therefore usually the limiting factor in the DNP-specific antibody response, rendering it a suitable means to assess Th cell responsiveness *in vivo* [25, 26]. Thus, DNP was covalently coupled to the N terminus of the LCMV GP-derived peptide P13 which in turn was covalently linked via its C terminus to BSA (DNP-P13-BSA). Importantly, the twofold modified P13 was still processed and efficiently presented on MHC class II molecules, as determined by *in vitro* activation of a P13-specific CD4$^+$ T cell hybridoma (not shown).

To address the question whether TCR transgenic CD4$^+$ T cells or LCMV-primed CD4$^+$ T cells of a C57BL/6 LCMV immune mouse were able to provide cognate help to DNP-specific B cells, TCR transgenic mice, naive C57BL/6 mice or LCMV-immune C57BL/6 mice were immunized intravenously with a limiting amount of DNP-P13-BSA. The DNP-specific IgG titers were determined

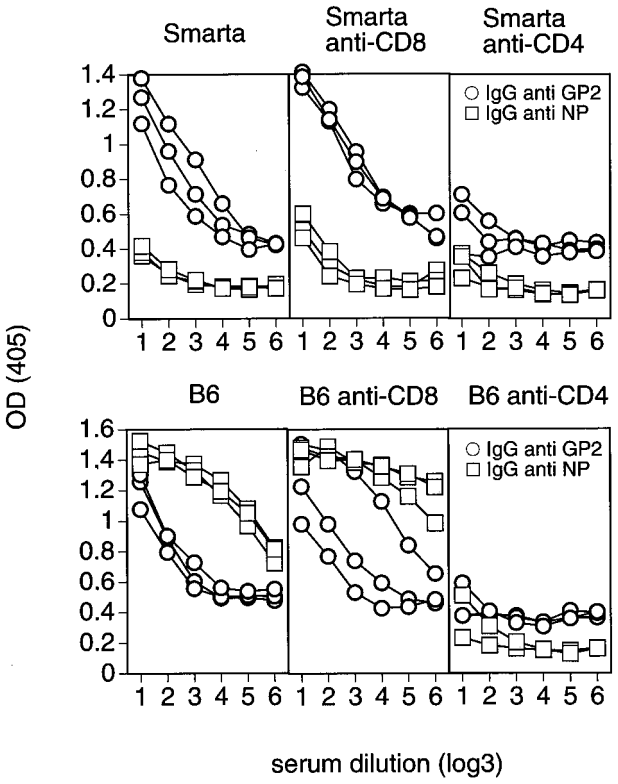

Figure 3. LCMV-specific IgG antibody responses. Untreated (left panels), CD8-depeleted (middle panels) or CD4-depleted (right panels) Smarta1 TCR transgenic mice (upper panels) or negative littermates (lower panels) were infected with 200 PFU LCMV. Eleven days later, LCMV GP2-specific (circles) or LCMV NP-specific (squares) IgG titers were determined in 30-fold prediluted sera by ELISA. Each line represents an individual mouse. One of four equivalent experiments is shown.

by ELISA 7 days after immunization (Fig. 4A). TCR transgenic mice exhibited high titers of DNP-specific IgG antibodies, whereas naive C57BL/6 mice only showed marginal induction of DNP-specific IgG antibodies. LCMV-immune nontransgenic mice showed enhancement of DNP-specific IgG production that was comparable to that of naive TCR transgenic mice, indicating that LCMV-specific memory T cell help may – at least in this experimental setup – be imitated by drastically elevated Th cell precursor frequencies. Furthermore, immunization of normal or TCR transgenic carrier mice with DNP-P13-BSA did not induce measurable DNP-specific IgG titers (Fig. 4B), indicating that TCR transgenic $CD4^+$ T cells were both physically and functionally absent in LCMV carrier mice.

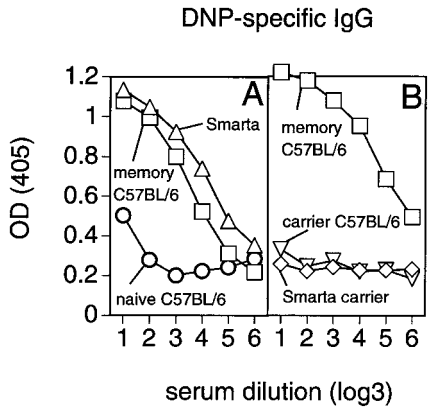

Figure 4. DNP-specific IgG antibody responses. Naive TCR transgenic mice, naive negative littermates and LCMV-immune C57BL/6 mice, TCR transgenic and nontransgenic carrier mice were immunized i.v. with 5 μg DNP-P13-BSA. Seven days after immunization, DNP-specific IgG titers were determined in 30-fold prediluted sera by ELISA on OVA-DNP-coated plates. Each line represents the mean of three individual mice. One of three comparable experiments is shown.

2.7 Assessment of cytotoxic T cell function in TCR transgenic mice

MHC class I-restricted CTL responses on day 8 after infection of the TCR transgenic mouse line Smarta1 were

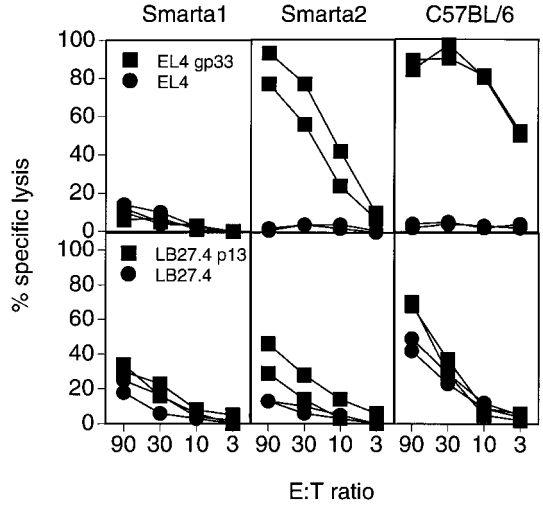

Figure 5. LCMV-specific cytotoxic activity *in vitro*. Smarta1 (left panels), Smarta2 (middle panels) transgenic mice and negative littermates (right panels) were infected with 200 PFU LCMV and CTL activity was determined 8 days after infection by ^{51}Cr release assay on the indicated targets: EL4 cells (MHC class II negative) and LB27.4 cells (MHC class II positive).

profoundly impaired, whereas the TCR transgenic mouse line Smarta2 showed reduced but nevertheless significant CTL activity when compared to negative littermates (Fig. 5, upper panels). This finding correlates well with the observation that the skewing of the CD4 : CD8 ratio towards the CD4 compartment is much more pronounced in Smarta1 than in Smarta2 transgenic mice (Fig. 1). Assessment of CTL activity 13 days after infection revealed low levels of MHC class I-restricted killing in Smarta1 transgenic mice, indicating delayed CTL responses in Smarta1 TCR transgenic mice (data not shown).

The high frequency and defined specificity of TCR transgenic $CD4^+$ T cells offered the unique possibility to address the question whether *ex vivo* isolated LCMV-specific $CD4^+$ T cells are able to specifically lyse MHC class II-positive target cells. Day 8 effector T cells from Smarta1 and Smarta2 transgenic mice and from negative littermates were assessed for their potential to lyse MHC class II^+Fas^+ LB27.4 target cells (lower panels) pulsed with the relevant MHC class II binding peptide P13. All mice showed very low but reproducible levels of specific lysis of P13-pulsed target cells; however, a marked nonspecific lysis of target cells was also observed for all effectors. Similar results were obtained for day 13 effector T cells from Smarta1 or Smarta2 TCR transgenic animals (data not shown). Note that LB27.4 cells pulsed with the D^b binding peptide gp33 were efficiently lysed by day 8 effector CTL from negative littermates (data not shown).

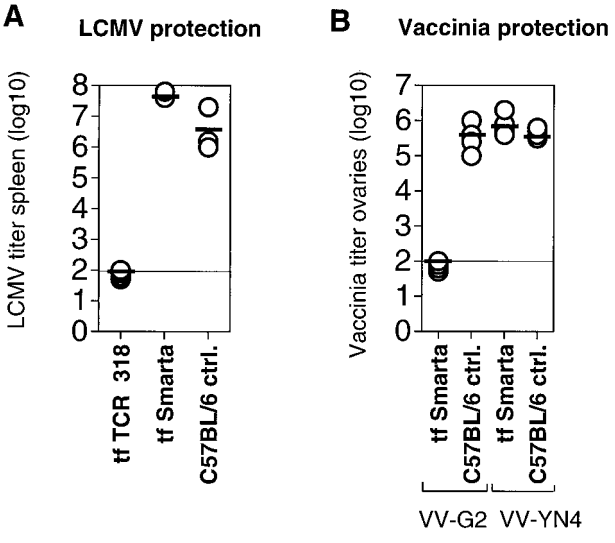

Figure 6. Protection against LCMV or recombinant vaccinia virus infection: (A) 1×10^7 *in vitro* activated $CD4^+$ TCR transgenic T cells from Smarta1 transgenic animals or 1×10^7 *in vitro* activated $CD8^+$ TCR transgenic T cells from 318 TCR transgenic animals [27] were adoptively transferred into naive C57BL/6 recipients and challenged intravenously with 200 PFU LCMV. After 4 days, LCMV titers were determined in the spleen. Each symbol represents an individual mouse. One of three comparable experiments is shown: (B) 1×10^7 activated $CD4^+$ T cells were transferred into naive C57BL/6 recipients and challenged with 2×10^6 PFU VV-G2 or VV-YN4. Vaccinia titers were determined in ovaries 5 days after infection. One of three comparable experiments is shown.

2.8 Protection from LCMV versus recombinant vaccinia virus replication

To compare protective capacities of LCMV-specific $CD4^+$ T cells and LCMV-specific $CD8^+$ T cells against LCMV infection, *in vitro* activated TCR transgenic $CD4^+$ or $CD8^+$ [27] T cells were adoptively transferred into naive C57BL/6 recipients and challenged i.v. with LCMV. Four days later, LCMV titers were determined in the spleen (Fig. 6A). Recipients of TCR transgenic $CD8^+$ T cells were fully protected whereas $CD4^+$ T cells did not confer protection. In line with this result, Smarta1 TCR transgenic animals (exhibiting virtually no $CD8^+$ T cell compartment) were unable to resolve LCMV infection whereas Smarta2 transgenic animals showed delayed LCMV clearance as compared to negative littermates (data not shown). Interestingly, transfer of activated TCR transgenic $CD4^+$ T cells fully protected C57BL/6 recipients from infection with recombinant vaccinia virus expressing the LCMV GP (VV-G2) (*i.e.* expressing the relevant epitope) but not from infection with recombinant vaccinia virus expressing the LCMV NP (VV-YN4) (Fig. 6B). These experiments establish, by using $CD4^+$ T cells of a single defined specificity, that different viruses are susceptible to distinct effector cells and mechanisms.

3 Discussion

3.1 Clonal deletion of TCR transgenic thymocytes in LCMV carrier mice

It has been shown using LCMV-specific MHC class I-restricted TCR transgenic mice that virus-specific $CD8^+$ T cells are negatively selected in the thymus of LCMV carrier mice [27]. However, much less was known about the fate of virus-specific $CD4^+$ T cells in such LCMV carrier mice. In fact, it has been argued that virus-specific $CD4^+$ T cells are present and functional in LCMV carrier mice based on the observation that LCMV carrier mice can exhibit high levels of LCMV-specific IgG titers [28–30]. However, LCMV-specific TCR transgenic $CD4^+$ T cells were negatively selected in the thymus of virus carrier mice leading to phenotypical and functional

absence of TCR transgenic $CD4^+$ T cells in the peripheral T cell population of LCMV carrier mice. These data suggest that – at least for this MHC class II-restricted TCR – LCMV-specific $CD4^+$ T cells are clonally deleted in the thymus and that the presence of LCMV-specific IgG titers may thus not be strictly linked to the presence of LCMV-specific $CD4^+$ T cells in LCMV carrier mice.

3.2 Functionality of TCR-transgenic T cells: antibody response

LCMV NP-specific IgG responses were virtually absent in TCR transgenic mice, whereas normal mice mounted a strong LCMV NP-specific IgG response. These results imply that Th cells exhibiting specificity for viral surface antigens are incapable of providing help for virus-internal proteins such as for LCMV NP. It has been shown for influenza and VSV infection, that Th cells with specificity for virus-internal proteins [such as the nucleoprotein (N) and the matrixprotein (M)] are able to mediate help for B cells with specificity for surface viral proteins [31–33]. This intrastructural help is apparently only functional for Th cells with specificity for virus internal proteins and B cells with specificity for virus external proteins but not vice versa. In the case of LCMV GP-specific TCR transgenic mice, the specificity of T help is strongly biased towards the LCMV GP and therefore LCMV NP-specific T help is virtually absent (which would be required for NP-specific B cell help), thus resulting in a reduction of LCMV NP-specific antibody production.

Interestingly, kinetics of LCMV GP-specific IgG production in TCR transgenic mice were not drastically enhanced as compared to normal mice, suggesting that during the normal LCMV-specific antibody response, B cells and not T helper cells are a limiting factor. Evidence for this hypothesis is given by the ability of TCR transgenic LCMV GP-specific $CD4^+$ T cells to provide enhanced help for hapten-specific B cells which are usually present at high precursor frequencies [22]; in this situation, TCR transgenic $CD4^+$ T cells enhanced IgG production by B cells. Although LCMV GP-specific transgenic $CD4^+$ T cells were able to provide cognate help for B cells *in vivo*, LCMV-neutralizing antibody responses (being specific for LCMV GP) could not be detected in TCR transgenic mice early after LCMV infection, indicating that the late appearance of neutralizing antibodies in normal mice after LCMV infection is not due to limiting T cell help.

3.3 Functionality of TCR-transgenic T cells: antiviral protection

This study clearly demonstrates that LCMV-specific $CD4^+$ T cells are incapable of mediating protection against LCMV infection despite the presence of very low and barely specific levels of *in vitro* MHC class II-restricted cytotoxicity. Protection against LCMV correlated with the presence of a sizable $CD8^+$ T cell compartment, the more $CD8^+$ T cells present the better and faster the clearance of the virus. This corroborates previous observations that clearance of LCMV infection is crucially dependent on perforin-mediated cytotoxicity by LCMV-specific $CD8^+$ T cells [2, 13, 14]. However, these previous studies could not exclude inefficient induction of LCMV-specific $CD4^+$ T cells upon LCMV infection and thus, the LCMV-specific $CD4^+$ TCR transgenic mice described in this study offered the opportunity to test adoptive transfer of activated $CD4^+$ TCR transgenic T cells for protection against LCMV. While adoptive transfer of activated $CD8^+$ TCR transgenic T cells protected against LCMV, adoptive transfer of activated transgenic $CD4^+$ T cells did not protect against LCMV. However, adoptive transfer of the identical, activated LCMV-specific $CD4^+$ T cells efficiently protected against infection with recombinant vaccinia virus expressing the relevant helper cell epitope. This is consistent with the finding that as opposed to the noncytopathic LCMV infection, cytopathic vaccinia virus infection is not controlled by perforin-mediated cytotoxicity but by either $CD4^+$ or $CD8^+$ T cell-dependent IFN-γ and TNF-α [34–36].

4 Materials and methods

4.1 Mice

Inbred C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Zuchthygiene (Tierspital Zürich, Switzerland). The generation of MHC class II-restricted TCR transgenic mice is described below. Mice were bred in specific pathogen-free facilities.

4.2 Viruses and recombinant proteins

The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube (Hamburg, Germany) and grown on L929 cells (ATCC CRL 1) with a low multiplicity of infection (MOI). Recombinant vaccinia viruses expressing LCMV GP or LCMV NP have been described [37, 38]. Recombinant viruses were grown at low MOI on BSC cells and plaqued on BSC cells. The recombinant baculovirus expressing LCMV NP has been described previously [39]. The recombinant bacteria expressing the LCMV GP2 protein have also been described previously [40].

4.3 Peptides

Peptides were purchased from NMR Reutlingen (Reutlingen, Germany) or from Neosystem (Strasbourg, France). The following peptides were used: P13: (GLNGPDIYKGVYQFKSVEFD) (LCMV-GP, I-A^b); P61: (SGEGWPYIACRTSVVGRAWE) (LCMV NP, I-A^b); and GP33: (KAVYNFATM) (LCMV GP, D^b).

4.4 Generation of TCR transgenic mice

4.4.1 Generation of an LCMV GP-specific $CD4^+$ T cell clone

C57BL/6 mice were infected intravenously with 200 PFU LCMV WE and 11 days later $CD4^+$ T cells were purified from single-cell spleen suspensions by MACS sorting according to the protocol of the supplier (Miltenyi Biotec, Germany) and were restimulated *in vitro* for several rounds with irradiated autologous spleen cells and the LCMV GP-derived P13 [41], finally leading to a LCMV-GP-specific $CD4^+$ T cell line. Limiting dilution of $CD4^+$ T cells yielded several LCMV-specific $CD4^+$ T cell clones, one of which (clone 1) was chosen for further characterization. Clone 1 was specific for the LCMV GP-derived P13 (amino acids 61–80) and restricted to the I-A^b MHC class II molecule. Sequencing of the TCR variable region genes revealed that the Vα variable region gene was composed of a Vα2.3-JαDK1 rearrangement and that the Vβ variable region gene was composed of a Vβ8.3-Jβ2.5 rearrangement (EMBL nucleotide sequence database, accession numbers Y11102 and Y11103)

4.4.2 Generation of the TCR transgenic mouse

The variable region genes of clone 1 were amplified by PCR using genomic DNA as a template and the following primer combinations for Vα2: 5'-TGACCCGGGAGCTTCAGTCTAGGAGGAATG-3' and 5'-TATGCGGCCGCTATCAGGTACTTACTGGGG-3'; for Vβ8.3: 5'-GGCCTCGAGCATACAAGAGCCTGACTTGGTCGCGAGATG-3' and 5'-TGCCCGCGGCTGAGAACGCGCACGTGGGG-3'.

The primers used for Vα2 variable gene amplification deliberately introduced a 5' flanking XmaI site (20 bp 5' of the ATG start codon) and a 3' flanking NotI site (30 bp 3' of the splice donor sequence of JαDK1). The primers used for Vβ8.3 variable gene amplification similarly introduced a 5' flanking XhoI site and a 3' flanking SacII site. After sequence analysis, the XmaI-NotI Vα2 fragment and the XhoI-SacII Vβ8.3 fragment were cloned into previously described genomic TCR expression cassette vectors [17]. The resulting Vα2-PαTRIS was digested with SalI to liberate a 17.5 kbp Vα2 injection fragment, whereas a 19 kbp Vβ8.3 injection fragment was excised from the resulting Vβ8.3-PβTRIS construct by digestion with KpnI. TCRαβ transgenic mice were generated at BRL (Biological Research Laboratories, Füllinsdorf, Switzerland) by coinjection of the Vα2 and Vβ8.3 fragments into fertilized C57BL/6 eggs yielding two TCR transgenic mouse lines (Smarta1 and Smarta2).

4.5 Cytofluorometric analyses

The following monoclonal antibodies were used for analysis of thymocytes and peripheral lymphocytes: biotinylated 7G8, specific for Vβ8.3 (a gift from Dr. I. Förster, [21]); phycoerythrin-conjugated B20.1, specific for Vα2 (purchased from Pharmingen); fluorescein-conjugated anti-CD8 (purchased from Gibco BRL) and fluorescein- or phycoerythrin-conjugated anti-CD4 (purchased from Gibco BRL). Tricolor-conjugated streptavidin or anti-CD4 were purchased from Caltag Laboratories. Flow cytometry was performed on a FACstar Plus flow cytometer (Becton Dickinson).

4.6 Proliferation

Purified $CD4^+$ T cells (6×10^4) from TCR transgenic mice or from negative littermates were incubated in flat-bottom 96-well plates with threefold serial dilutions of LCMV (MOI = 2 at the highest concentration), LCMV GP-derived P13 (1 μg/ml at the highest concentration) or LCMV NP-derived P61 (1 μg/ml at the highest concentration) in the presence of 6×10^5 irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. Proliferation was assessed by incorporation of [^{3}H] thymidine (1 μCi/well).

4.7 Cytokine analysis

Supernatants of proliferation assays as described above were analyzed for IL-2 content (24 h after restimulation), IFN-γ content (60 h after restimulation) and IL-4 content (60 h after restimulation). IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue™ color reaction (Biosource International) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore) fluorimeter. IFN-γ and IL-4 were assessed by ELISA as described [42, 43].

4.8 Adoptive transfer of TCR transgenic T cells

MHC class II-restricted $CD4^+$ TCR transgenic T cells from Smarta1 transgenics or MHC class I-restricted $CD8^+$ TCR transgenic T cells from 318 transgenics [27] were activated *in vitro* with the relevant peptides for 4 days. TCR transgenic T cells (10^7) were adoptively transferred into naive C57BL/6 recipients and challenged either intravenously with 200 PFU LCMV or intraperitoneally with 2×10^6 PFU recombinant vaccinia virus. Viral titers were determined in the spleen 4 days after challenge with LCMV or in the ovaries 5 days after challenge with recombinant vaccinia virus.

4.9 ELISA

The LCMV NP-specific ELISA has been described previously [44]. ELISA measurement of LCMV-GP-specific or DNP-specific IgG titers was performed similarly. ELISA plates were either coated with 0.1 μg/well purified recombinant LCMV-GP2 or with 0.1 μg/well OVA covalently coupled to DNP [25, 45].

In vivo depletion of $CD4^+$ or $CD8^+$ T cells was achieved by administration of the depleting monoclonal antibody YTS 169.4 or YTS 191.1, respectively [46].

4.10 Cytotoxic T cell response

Ex vivo cytolytic activity of spleen cells was determined as previously described [47]. TCR transgenic mice were infected intravenously with 200 PFU LCMV and 8 or 13 days after infection, spleen cell suspensions were prepared and tested on peptide-pulsed (gp33: 1 μM; P13 100 μM) EL4 ($H\text{-}2^b$) or LB27.4 ($H\text{-}2^{d/b}$, MHC class II^+ Fas^+) cells.

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