IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Endogenous Il10 Alleviates the Systemic Antiviral Cellular Immune Response and T Cell—Mediated Immunopathology in Select Organs of Acutely LCMV-Infected Mice

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The immunoregulatory cytokine IL-10 suppresses T-cell immunity. The complementary question, whether IL-10 is also involved in limiting the collateral damage of vigorous T cell responses, has not been addressed in detail. Here, we report that the particularly strong virus-specific immune response during acute primary infection with the lymphocytic choriomeningitis virus (LCMV) in mice is significantly further increased in Il10—deficient mice, particularly regarding frequencies and cytotoxic activity of CD8⁺ T cells. This increase results in exacerbating immunopathology in select organs, ranging from transient local swelling to an increased risk for mortality. Remarkably, LCMV-induced, T cell—mediated hepatitis is not affected by endogenous Il10. The alleviating effect of Il10 on LCMV-induced immunopathology was found to be operative in delayed-type hypersensitivity footpad-swelling reaction and in debilitating meningitis in mice of both the C57BL/6 and BALB/c strains. These strains are prototypic counterpoles for genetically imprinted type 1-biased versus type 2-biased T cell—mediated immune responses against various infectious pathogens. However, during acute LCMV infection, neither systemic cytokine patterns nor the impact of Il10 on LCMV-induced immunopathology differed conspicuously between these two strains of mice. This study documents a physiological role of Il10 in the regulation of a balanced T-cell response limiting immunopathological damage. (*Am J Pathol* 2015, 185: 3025–3038; <http://dx.doi.org/10.1016/j.ajpath.2015.07.019>)

The cytokine IL-10 is an immunoregulatory master switch that inhibits innate as well as adaptive immune responses (reviewed by Moore et al¹ and O'Garra et al²). Il10 has been reported to limit virus-specific immune responses during acute infection, such as with influenza virus,^{3,4} herpes simplex virus,⁵ and murine cytomegalovirus.⁶ Similarly, Il10 contributes to inhibiting virus-specific immunity during persistent viral infections, such as with murine cytomegalovirus^{7,8} or lymphocytic choriomeningitis virus (LCMV).^{9,10} In particular, in mice persistently infected with LCMV, abolishing Il10 activity leads to restoring an effective immune response that eliminates the virus.^{9,10} Consequently, therapeutic strategies to block the

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activity of II10 are discussed and explored for the treatment of widespread persistent viral infection in humans, such as chronic infection with hepatitis B virus or HIV.¹¹

The downside of such therapeutic approaches would be the risk for promoting overshooting immune reactions by unleashing virus-specific effector cells. Surprisingly, the adverse effects of II10 inhibition in the murine model of LCMV infection have not yet been addressed. The present study aims at quantitatively characterizing the sequelae of inhibiting II10 during LCMV-induced immunopathology. The organ-specific pathology is mediated by virus-specific CD8⁺ and CD4⁺ T lymphocytes and, thus, represents antigen-specific damage rather than collateral damage.

We report that a deficiency in II10 further increases the already vigorous T cell-mediated immune response against LCMV, indeed accelerating virus elimination. However, the flipside of these effects is enhanced virus-induced immunopathology.

We observed that II10-mediated immunoinhibitory activity is operative in both C57BL/6 and BALB/c—prototypic mouse strains of antipodal immunoregulatory activity characterized by type 1-biased versus type 2-biased cytokine milieu, respectively, in a variety of infectious diseases.^{12–14} We report a differential impact of II10 on organ-specific immunopathology. Specifically, II10 alleviates the LCMV-induced delayed-type hypersensitivity (DTH) footpad-swelling reaction as well as life-threatening choriomeningitis, whereas the fulminant viral hepatitis is not affected.

Taken together, these data show that II10 is crucial for immunoregulation and for immunopathology in select tissues and organs during acute LCMV infection.

Materials and Methods

Mice and Virus

For this study, the following lines of mice were used: II10-deficient (*II10*^{−/−}) mice of the C57BL/6 strain (kindly provided by Dr. Werner Müller, Institute for Genetics, Cologne, Germany) and of the BALB/c strain (a kind gift from Dr. Donna Rennick, DNAX, Palo Alto, CA)¹⁵; perforin-deficient mice (*Perf*^{−/−}; kindly donated by Dr. Hans Hengartner, University of Zürich, Zürich, Switzerland)¹⁶; mice with LoxP-flanked alleles of *II10* (*II10*^{fl/fl}; C57BL/6, kindly provided by Dr. Werner Müller; BALB/c, kindly provided by Dr. Axel Roers, Technical University, Dresden, Germany)¹⁷; mice expressing Cre recombinase under control of the *CD4* promoter (*CD4Cre*^{pos}; a kind gift from Dr. Axel Roers)¹⁸ or of the *CD11c* promoter (*CD11cCre*^{pos}; kindly donated by Dr. Boris Reizis, Columbia University, New York, NY).¹⁹ Mice were genotyped by end-point PCR analysis of tail biopsy samples, as published.^{15–19} All strains of mice were backcrossed at least 10 times to either the C57BL/6 or the BALB/c background. The mice were bred and kept strictly under barrier conditions and used when 8 to 12 weeks old. Experiments were performed in accordance with the Animal Protection

Law of Germany after approval by the Ethics Committee at the University of Cologne.

Throughout this study, the WE strain of LCMV was propagated and titrated as plaque-forming units on L929 cells, as described.²⁰ When applicable, plaque-forming units were converted to mouse-infectious units (IU) by multiplication with the factor of 10, as published.²¹ Mice were infected with 10⁵ IU i.v., 10⁶ i.p., 10³ IU intracerebrally (i.c.), or 10⁵ IU s.c. in the hind footpad, as indicated in the respective experiments. For the quantification of organ virus concentrations, weighed portions of organs were homogenized in phosphate-buffered saline and supplemented with 1% fetal calf serum and penicillin/streptomycin, and plaque-forming units on L929 cells were determined.

Chromium-Release Assay

Cytotoxic T-lymphocyte activity was measured according to Brunner et al,²² with modifications.²³ Briefly, effector-cell populations were either single-cell suspensions of complete spleen cells or immunomagnetically enriched (MACS; Miltenyi Biotec, Gladbach, Germany) CD8⁺ lymphocytes (purity >95% as determined by flow cytometry). These effector cells were incubated at graded densities with LCMV-infected or noninfected ⁵¹Cr-labeled SV40-transformed fetal fibroblastic C57BL/6-SV or BALB/c-SV target cells at 37°C. After 4 hours, ⁵¹Cr release was measured, and the percentage of lysis was calculated as:

$$\% \text{ Lysis} = \left(\frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \right) \times 100, \quad (1)$$

where *cpm* is counts per minute.

Flow Cytometry

Flow cytometry of splenic lymphocytes was performed according to standard procedures, using fluorescent monoclonal antibody (mAb) specific for CD4 (GK 1.5) or CD8α (53–6.7) on a FACSCalibur cytometer (BD Biosciences, San Jose, CA). LCMV-specific CD8⁺ T cells were detected among the spleen cells of C57BL/6 or BALB/c mice by using phycoerythrin-conjugated H-2D^b-dextramers loaded with either of the three H-2D^b-restricted immunodominant epitopes of LCMV (GP_{33–41}, GP_{276–286}, or NP_{396–404}) or with the H-2L^d-restricted immunodominant epitope of LCMV (NP_{118–126}), respectively, according to the instructions of the manufacturer (Immudex, Copenhagen, Denmark).

CD4⁺ T-Cell Depletion

CD4-specific mAbs were affinity-purified from supernatants of the rat hybridomas YTS191.1 and YTA3.1.^{24,25} A mixture of 250 μg of each mAb was injected i.p. into mice on days −4, −1, 0, and +1 related to the day of infection (day 0). Control mice were treated with purified rat IgG (Sigma-Aldrich, Taufkirchen, Germany). Depletion efficacy

Figure 1 Effect of IL10 deficiency on the splenic immune response in C57BL/6 and BALB/c mice acutely infected with lymphocytic choriomeningitis virus (LCMV). **A–H:** *Il10*^{-/-} mice or wild-type (wt) littermates of the strains C57BL/6 (**A**, **C**, **E**, and **G**) and BALB/c (**B**, **D**, **F**, and **H**) were infected with 10⁵ mouse-infectious units (IU) of LCMV strain WE i.v. On the indicated days of infection, spleens were harvested to prepare organ homogenates (**A** and **B**) or single-cell suspensions (**C–H**). **A** and **B:** Viral loads in the spleens on days 4, 6, and 8 of infection. **C** and **D:** LCMV-specific cytolytic activity of total splenocytes in 4-hour ⁵¹Cr release assays against LCMV-infected syngeneic fibroblasts on day 8 of infection (**left panels**). Percentages of CD8⁺ cells among total lymphatic splenocytes were determined by flow cytometry (**right panels**). **E** and **F:** LCMV-specific cytolytic activity of immunomagnetically enriched CD8⁺ spleen cells (purity >95%) against LCMV-infected syngeneic fibroblasts on day 8 of infection. **G** and **H:** Scatterplots show representative examples of staining for H-2 D^b/GP₃₃₋₄₁ (**G**) and H-2 L^d/NP₁₁₈₋₁₂₆ (**H**) (**left panels**). Percentages of LCMV-specific CD8⁺ T cells within the effector-cell populations used in **C** and **D**. LCMV-specific CD8⁺ T cells were detected by flow cytometry with major histocompatibility complex (MHC) dextramers loaded with LCMV-specific epitopes [MHC class I alleles and epitopes: H-2 D^b-restricted immunodominant epitopes GP₃₃₋₄₁, GP₂₇₆₋₂₈₆, and NP₃₉₆₋₄₀₄ (**G**), H-2 L^d-restricted immunodominant epitope NP₁₁₈₋₁₂₆ (**H**)]. Percentages of CD8⁺ spleen cells expressing T-cell receptors (TCRs) for the three major immunodominant H-2 D^b-restricted epitopes GP₃₃₋₄₁, GP₂₇₆₋₂₈₆, and NP₃₉₆₋₄₀₄ (**G**) and the immunodominant H-2 L^d-restricted epitope NP₁₁₈₋₁₂₆ (**H**) (**right panels**). Data are expressed as means ± SEM cumulative log₁₀ values [in plaque-forming units (PFU) per gram] from two experiments on days 4 and 6 of infection (**A** and **B**); from four (**F**) and five (**C–E**) experiments; from nine experiments on day 8 of infection (**A**); from 12 experiments on day 8 of infection (**B**); and as indicated in **C** and **D** (**G** and **H**). *n* = 3 (**B**, mice per genotype on days 4 and 6 of infection); *n* = 4 (**A**, mice per genotype on days 4 and 6 of infection); *n* = 8 (**C**, mice per genotype); *n* = 11 (**D**, mice per genotype; **F**, total mice per genotype); *n* = 13 (**E**, total mice per genotype); *n* = 20 (**A**, mice per genotype on day 8 of infection); *n* = 30 (**B**, mice per genotype on day 8 of infection). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 [pair-wise comparisons using the two-sample *t*-test for independent samples (two-sided)].

manufacturer's instructions (Roche Diagnostics Deutschland GmbH, Mannheim, Germany).

Additionally, livers were prepared for histopathological analysis. After 24 hours of fixation in 4% buffered formalin, tissues were embedded into paraffin. Hepatic fibrosis was assessed on sections after Sirius staining. The inflammatory activity [ie, periportal or periseptal interface hepatitis, confluent necrosis, focally (spotty) lytic necrosis, apoptosis, focal inflammation, and portal inflammation] was assessed according to the Ishak score in hematoxylin and eosin-stained samples.²⁷

Clinical Course and Histopathology of LCMV

After injection of LCMV 10^3 IU i.c., mice were monitored for clinical symptoms daily over the indicated observation periods.

For brain histopathology and immunohistochemistry analysis, mice were intracardially perfused with NaCl while under deep CO₂ anesthesia. Brains were prepared and processed to yield frozen and paraffin sections, as described.²⁸ Paraffin sections were stained with hematoxylin and eosin and with Luxol fast blue with cresyl violet, respectively. Immunohistochemistry analysis was performed on cryopreserved specimens by use of the avidin-biotin complex technique with appropriate biotinylated secondary antibodies (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA). Peroxidase reaction product was visualized using 3,3-diaminobenzidine (Sigma-Aldrich) as chromogen and H₂O₂ as cosubstrate, with the following monoclonal rat anti-mouse antibodies: CD8 α (clone 53-6.7), CD45 (clone M1/9.3.4.HL.2), and major histocompatibility complex class II antigen (I-Ab,d,q haplotypes; clone M5/114.15.2).

For semiquantitative evaluation of meningitis and ventriculitis, a grading system was introduced, as follows: 0, no infiltrates; +, single inflammatory cells; ++, small infiltrates of inflammatory cells, not exceeding one layer of lymphocytes; and +++, large infiltrates of inflammatory cells, exceeding one layer of lymphocytes. The slides were assessed independently by two pathologists (A.B. and M.D.) in a blinded fashion.

Quantification of Cytokines in Sera

In serum samples, concentrations of Il10, interferon (Ifn)- γ , Il4, and tumor necrosis factor (Tnf) were quantified either by specific enzyme-linked immunosorbent assay (R&D Systems, Abingdon, UK) or by the LEGENDplex Mouse Th1/Th2 Panel (BioLegend, San Diego, CA) according to the instructions of the manufacturers.

Statistical Analysis

Data on continuous variables were summarized by means $\pm$ SD or SEM. Groups were compared using the two-sample *t*-test for independent samples (two-sided). Figure legends report the number of mice studied per group.

Serial measurements of the intensity of the DTH reaction, or Tnf α and Ifn γ concentrations, were analyzed using the area under the curve as a summary measure. Area under the curve was divided by the individual duration of the measurement period to account for different observation times due to early deaths. Data are presented by course of means over time.

Survival times after infection are displayed using Kaplan-Meier curves. Groups were compared using the log-rank test, and point-wise 95% CIs for survival probability were calculated.

A two-sided probability value of <0.05 was considered statistically significant, although no adjustment for multiplicity was made. Statistical analyses were performed using the IBM SPSS Statistics software package version 22.0 (IBM Corp., Armonk, NY).

Results

Il10 Deficiency Enhances CD8⁺ T-Cell Responses and Accelerates Virus Elimination during Acute LCMV Infection

Antigen-specific T-cell responses are generally enhanced by type 1-biased and inhibited by type 2-biased T cell-derived cytokine milieus present during the induction and maturation of the effector cells. In some infection

Figure 2 Systemic cytokine pattern and influence of CD4⁺ T cells on the expansion and cytotoxic activity of lymphocytic choriomeningitis virus (LCMV)-specific CD8⁺ T cells in *Il10*^{-/-} mice. Mice were infected with 10^5 mouse-infectious units (IU) of LCMV strain WE i.v. **A–D:** Th1 versus Th2 lead cytokines were quantified in the serum of wild-type (wt) C57BL/6 and BALB/c mice. Concentrations of interferon (Ifn)- γ (**A**), tumor necrosis factor (Tnf) (**B**), Il4 (**C**), and Il10 (**D**). **E–L:** From *Il10*^{-/-} and wt littermates of the BALB/c strain, spleens were harvested to prepare single-cell suspensions on the indicated days of infection. The percentages of CD4⁺ cells (**E**), total CD8⁺ cells (**F**), and LCMV-specific T-cell receptors (TCR_{LCMV}) CD8⁺ cells among lymphatic spleen cells (**G**) were determined by flow cytometry. **H and J:** Groups of mice were depleted of CD4⁺ T cells by i.p. injection of 500 μ g CD4-specific monoclonal antibody or control (Ctrl) with normal rat IgG on days -4, -1, 0, and +1 related to the day of infection (day 0). On day 8 of infection, LCMV-specific cytolytic activity of total spleen cells (**H**) or of immunomagnetically enriched CD8⁺ spleen cells (purity >95%) (**J**) was determined in 4-hour ⁵¹Cr release assays against LCMV-infected BALB/c fibroblasts. **I:** The percentage of CD8⁺ T cells among lymphatic spleen cells was determined by flow cytometry. **K:** Percentages of TCR_{LCMV} CD8⁺ T cells among lymphatic spleen cell populations were determined by flow cytometry with H-2 L^d major histocompatibility complex (MHC)-dextramers loaded with the LCMV-derived immunodominant epitope NP₁₁₈₋₁₂₆. **L:** Homogenates were prepared from segments of the spleens used in **I** to determine the viral load in the spleens on day 8 of infection. Data are expressed as means $\pm$ SEM cumulative values from two (**A–C**), three (**K**), or four (**D** and **I**) experiments; as cumulative values from two experiments on days 0 to 6 of infection and from three experiments on day 8 of infection (**E–G**); as means $\pm$ SEM values from four experiments (**H** and **J**); and as means $\pm$ SEM log₁₀ values [in plaque-forming units (PFU) per gram] (**L**). *n* $\geq$ 3 (**A–D**, mice per day and experiment on days 3, 4, and 6 of infection); *n* = 4 (**E–G**, mice per genotype on days 0 to 6 of infection; **H** and **J**, control-treated *Il10*^{-/-} and wt mice); *n* $\geq$ 4 (**A–D**, mice per day and experiment on days 0 and 8); *n* = 5 (**H** and **J**, anti-CD4-treated wt mice); *n* = 6 (**E–G**, mice per genotype on day 8 of infection; **K**, control-treated *Il10*^{-/-} and wt mice); *n* = 8 (**H** and **J**, anti-CD4-treated *Il10*^{-/-} mice); *n* = 9 (**I**, control-treated *Il10*^{-/-} and wt mice; **K**, anti-CD4-treated *Il10*^{-/-} and wt mice); *n* = 11 (**I**, anti-CD4-treated *Il10*^{-/-} and wt mice). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 [pair-wise comparisons using the two-sample *t*-test for independent samples (two-sided)].

Figure 3 Identification of the cellular source of IL10-dampening lymphocytic choriomeningitis virus (LCMV)-specific CD8⁺ T-cell responses. C57BL/6 (A and C) or BALB/c (B and D) mice were infected with 10⁵ plaque-forming units (PFU) of LCMV strain WE i.v. LCMV-specific cytolytic activity of splenic single-cell suspensions prepared from mice with cell type-specific deficiency of IL10 in T lymphocytes (*Il10^{fl/fl}/CD4Cre^{pos}*; A and B) or in dendritic cells (*Il10^{fl/fl}/CD11cCre^{pos}*; C and D) and their respective *Il10^{fl/fl}/CD4Cre^{neg}* littermates. Shown is the specific cytolytic activity of total spleen cells or immunomagnetically enriched CD8⁺ spleen cells (purity of CD8⁺ cells >95%) as determined in 4-hour ⁵¹Cr release assays against LCMV-infected syngeneic fibroblasts on day 8 of infection. Additionally, splenic viral loads were determined. Data are expressed as means ± SEM cumulative values from three (B and C), four (D), or five experiments (A). *n* = 5 (D, *Il10^{fl/fl}/CD11cCre^{neg}*); *n* = 6 (B, *Il10^{fl/fl}/CD4Cre^{neg}*; C, *Il10^{fl/fl}/CD11cCre^{neg}*; D, *Il10^{fl/fl}/CD11cCre^{pos}*); *n* = 7 (C, *Il10^{fl/fl}/CD11cCre^{pos}*); *n* = 10 (B, *Il10^{fl/fl}/CD4Cre^{pos}*); *n* = 14 (A, *Il10^{fl/fl}/CD4Cre^{neg}* and *Il10^{fl/fl}/CD4Cre^{pos}*). **P* < 0.05, ***P* < 0.01 (pair-wise comparisons between *Cre^{pos}* and *Cre^{neg}* mice using the two-sample *t*-test for independent samples).

models, C57BL/6 mice have been identified as type 1-biased responders, whereas BALB/c mice are more prone to type 2-biased immune responses.^{12–14} Therefore, the influence of IL10 on the course and strength of the LCMV-induced, T cell-mediated immunopathology was characterized in the *Il10*^{-/-} mice of both the C57BL/6 and the BALB/c strains.

In the C57BL/6 and BALB/c *Il10*^{-/-} mice infected with LCMV strain WE 10⁵ IU i.v., the kinetics of virus elimination were significantly accelerated as compared with those in the respective wild-type (wt) littermates (Figure 1, A and B).

Because virus-specific CD8⁺ T cells are necessary and sufficient for eliminating LCMV during acute primary infection,²⁹ the effects of IL10 on CD8⁺ T cells were characterized in greater detail. The LCMV-specific cytotoxic activity of total spleen cells from the C57BL/6 or BALB/c *Il10*^{-/-} mice was significantly stronger than that of total spleen cells from the respective wt littermates (Figure 1, C and D). This increase was about twofold stronger in the BALB/c than in the C57BL/6 mice, as assessed using the

effector/target ratios required for achieving a defined degree of lysis (Figure 1, C and D).

The cytotoxic activity of total spleen cell preparations depends on the percentage of CD8⁺ T cells among total spleen cells and on the percentage of LCMV-specific T cells within the population of CD8⁺ T cells. In both mouse strains, the enhanced LCMV-specific cytotoxic activity of total spleen cells from the *Il10*^{-/-} mice was accompanied by a significantly increased percentage of CD8⁺ T cells among the spleen cells on day 8 of infection (Figure 1, C and D).

The LCMV-specific cytotoxicity of enriched CD8⁺ T cells was not altered in the C57BL/6 *Il10*^{-/-} mice compared with the wt mice (Figure 1E). In contrast, enriched CD8⁺ T cells from the BALB/c *Il10*^{-/-} mice exerted substantially greater specific cytotoxicity than did the corresponding wt control CD8⁺ T cells (Figure 1F).

The percentages of cells with T-cell receptors specific for LCMV (TCR_{LCMV}⁺) among CD8⁺ T cells did not differ in spleens of the C57BL/6 *Il10*^{-/-} mice versus the wt mice (Figure 1G). In contrast, the BALB/c *Il10*^{-/-} mice had substantially greater percentages of TCR_{LCMV}⁺ cells among CD8⁺

Figure 4 Effect of Il10 deficiency on the lymphocytic choriomeningitis virus (LCMV)-specific delayed-type hypersensitivity reaction. *Il10*^{-/-} mice (closed circles) or wild-type (wt) littermates (open circles) of the C57BL/6 (A) or BALB/c (B) strain were infected by s.c. injection of 10⁵ mouse-infectious units (IU) of LCMV strain WE into the right hind footpad. Footpad swelling was measured at 24-hour intervals and is shown as the swelling factor with which the dorsoventral thickness of the inoculated foot exceeded that of the contralateral, non-inoculated foot. Serial measurements on the footpad swelling were analyzed using the area under the curve (AUC) as a summary measure. Data are expressed as cumulative values from three (A) or four (B) experiments. *n* = 12 (A, *Il10*^{-/-}); *n* = 15 (A, wt); *n* = 17 (B, wt); *n* = 19 (B, *Il10*^{-/-}). **P* < 0.05 (specific values: C57BL/6, *P* = 0.012; BALB/c, *P* = 0.017; two-sample *t*-test).

cells in spleens than did their wt littermate controls (Figure 1H). Biologically most relevant are the frequencies of TCR_{LCMV}⁺ CD8⁺ T cells among total splenic lymphocytes, because these frequencies directly correlate with the probabilities of encounters between LCMV-specific effector cells and LCMV-infected target cells. Calculations according to the formula:

$$\left(\text{Percentage of CD8}^+ \text{ spleen cells} \right) \times \left(\text{Percentage of TCR}_{\text{LCMV}}^+ \text{ CD8}^+ \text{ T cells} \right) / 100 \quad (2)$$

revealed that in the *Il10*^{-/-} mice of both strains, the frequencies of TCR_{LCMV}⁺ CD8⁺ T cells among total spleen cells are greater than those in the respective wt control mice and that frequencies are 1.5- to 2-fold greater in the C57BL/6 mice than in the corresponding BALB/c genotype (C57BL/6 mice: *Il10*^{-/-}, approximately 12.8%; wt, approximately 8.7%; BALB/c mice: *Il10*^{-/-}, approximately 9.3%; wt, approximately 4.6%).

All together, increased frequencies of TCR_{LCMV}⁺ CD8⁺ T cells are accompanied in spleens of *Il10*^{-/-} mice of the C57BL/6 as well as the BALB/c strains by accelerated virus clearance during acute LCMV infection as compared to the respective wt controls.

CD4⁺ T Cells Support Expansion and Cytotoxic Activity of LCMV-Specific CD8⁺ T Cells in *Il10*^{-/-} Mice

In acute LCMV infection of mice, CD4⁺ T cells are not essential for the generation of an effective virus-specific immune response.²⁹ However, CD4⁺ T cells are strongly activated and support the generation of LCMV-specific memory CD8⁺ T cells (reviewed by Wiesel and Oxenius³⁰). During acute LCMV infection, the pattern of the prototypic Th1 cytokines, Ifnγ and Tnf (Figure 2, A and B),

versus Th2 cytokines, Il4 and Il10 (Figure 2, C and D), did not allow for the classification of C57BL/6 mice as typical Th1 responders versus BALB/c mice as typical Th2 responders, as reported for various infections.^{12–14,31} On the contrary, serum levels of Ifnγ were much greater in the BALB/c mice than in the C57BL/6 mice during the early days of infection (Figure 2A). Remarkably, the greatest serum levels of Il10 were detected in both strains of mice as early as 2 days of infection (Figure 2D).

CD4⁺ T-helper cells are major producers of the immunoregulatory cytokine Il10. This raised the question of whether the enhancing effect of deleting the single cytokine Il10 might obviate the helper effect of CD4⁺ T cells on the generation of strong, LCMV-specific, CD8⁺ T cell-mediated immune responses. Experiments addressing this question were performed in BALB/c mice after we observed a more pronounced effect of Il10 on the LCMV-specific response of spleen cells from this mouse strain (Figure 1).

CD4⁺ T cells were present at similar frequencies in the spleens of the BALB/c *Il10*^{-/-} and wt mice from days 0 to 6 of infection with LCMV (Figure 2E). However, between days 6 and 8 of infection, the frequencies of CD4⁺ T cells dropped significantly in the *Il10*^{-/-} mice, to about 40% less than those in the wt mice. This finding is in contrast to those of CD8⁺ T cells (Figure 2F) and LCMV-specific CD8⁺ T

Figure 5 Hepatic lesions in *Il10*^{-/-} and wild-type (wt) mice on day 8 of infection with lymphocytic choriomeningitis virus (LCMV) i.p. *Il10*^{-/-} mice (A and C) or wt littermates (B and D) of the C57BL/6 (A and B) or BALB/c (C and D) strain were infected by injection with LCMV strain WE 10⁶ mouse-infectious units (IU) i.p. On day 8 of infection, serial sections of the liver were subjected to either standard hematoxylin and eosin (HE) staining or to immunostaining with CD45-specific monoclonal antibodies (insets). Scale bars: 200 μm.

cells (Figure 2G), the frequencies of which were greater in the spleens of the BALB/c *Il10*^{-/-} mice than in those of the wt mice. Thus, there is divergence in the population dynamics of splenic CD4⁺ and CD8⁺ T cells in BALB/c *Il10*^{-/-} mice compared with those in their wt littermates. We asked whether CD4⁺ T cells have a particular impact on the effector functions of CD8⁺ T cells in LCMV-infected *Il10*^{-/-} mice, and we depleted the CD4⁺ T cells in those mice. Depletion of CD4⁺ T cells reduced the LCMV-specific cytolytic activity of total spleen cells from the BALB/c *Il10*^{-/-}

and the wt mice on day 8 of infection (Figure 2H). The reduced cytolytic activity did not coincide with reduced frequencies of CD8⁺ T cells in total spleen cells (Figure 2I). However, it has to be considered that the depletion of CD4⁺ T cells per se skews the proportion of CD8⁺ T cells among total spleen cells to greater percentages. This impediment in the quantitative assessment of CD8⁺ T-cell activity was bypassed by further analysis of immunomagnetically enriched CD8⁺ T cells.

The LCMV-specific cytolytic activity of the enriched CD8⁺ T cells from the *Il10*^{-/-} and wt mice depleted of

CD4⁺ T cells was significantly reduced compared with those in the respective control-treated littermates (Figure 2J). Concurrently, in both the *Il10*^{-/-} and the wt mice, the depletion of CD4⁺ T cells was associated with a significantly reduced frequency of splenic TCR_{LCMV}⁺ CD8⁺ T cells to about 50% of the levels observed in the respective control-treated littermates (Figure 2K). Collectively, with regard to CD8⁺-enriched spleen cells, cytolytic activity and percentages of LCMV-specific CD8⁺ T cells concordantly were greatest in the control-treated *Il10*^{-/-} mice. Both parameters reached similar intermediate levels in both the CD4⁺ T cell-depleted *Il10*^{-/-} mice and the control-treated wt mice, whereas the lowest levels were detected in the CD4⁺ T cell-depleted wt mice.

Despite its effect on the expansion and *ex vivo* cytolytic activity of LCMV-specific CD8⁺ T cells, the depletion of CD4⁺ T cells did not affect the viral load in the spleens of the *Il10*^{-/-} mice by day 8 of infection (Figure 2L). In contrast, in the wt mice it resulted in a significantly greater viral load than that in the control-treated littermates.

Taken together, although Il10 deficiency diminishes the splenic CD4⁺ T-helper cell population during acute LCMV infection, the direct impact of Il10 deficiency on CD8⁺ T cells overrides a possible effect of this reduction in T-helper cell frequencies.

Identification of the Il10-Producing Cell Type Crucial for Down-Modulating the LCMV-Specific Immune Response

Systemic levels of Il10 were detected at peak levels already at 2 days of infection and slowly declined thereafter in both the C57BL/6 and the BALB/c mice (Figure 2D). For a thorough understanding of the Il10-dependent immunoregulatory circuits in acute LCMV infection, we aimed at identifying the Il10-producing cell type(s) that suppress the LCMV-specific CD8⁺ T cell response.

Various myeloid and lymphoid cell types have been shown to produce Il10 during acute and chronic LCMV infection.³² During acute LCMV infection, assumedly the two candidate cell types that produce Il10 are T lymphocytes and dendritic cells

(DCs),^{33–35} because T cells are the effector cells necessary and sufficient for control of the virus, and DCs are necessary for inducing virus-specific T cells. To assess the effect of Il10 produced by either T cells or DCs, we used LoxP/Cre technology to generate mice with cell type-specific deficiency in Il10.

In the C57BL/6 strain, T cell-specific Il10 deficiency (C57BL/6 *Il10*^{fl/fl}/CD4Cre^{pos}) was associated with increased LCMV-specific cytolytic activity of total spleen cells compared with that in the corresponding wt controls (C57BL/6 *Il10*^{fl/fl}/CD4Cre^{neg}; Figure 3A). This difference was almost abolished when immunomagnetically enriched splenic CD8⁺ T cells were used as effector cells (Figure 3A). In contrast, in the BALB/c strain, both total spleen cells as well as CD8⁺-enriched effector cells from the BALB/c *Il10*^{fl/fl}/CD4Cre^{pos} mice exerted significantly greater cytotoxicity than did cells from BALB/c *Il10*^{fl/fl}/CD4Cre^{neg} littermates (Figure 3B). The splenic viral load was significantly lower in *Il10*^{fl/fl}/CD4Cre^{pos} mice than in *Il10*^{fl/fl}/CD4Cre^{neg} controls of both the C57BL/6 and BALB/c strains (Figure 3, A and B).

In the C57BL/6 strain, DC-specific Il10 deficiency (C57BL/6 *Il10*^{fl/fl}/CD11cCre^{pos}) did not affect the LCMV-specific cytolytic activity of either total or CD8⁺ T cell-enriched spleen cells (Figure 3C). In contrast, both total as well as CD8⁺ T cell-enriched spleen cells from BALB/c mice with DC-specific Il10 deficiency (BALB/c *Il10*^{fl/fl}/CD11cCre^{pos}) were associated with significantly stronger LCMV-specific cytotoxicity than were the respective effector cells from the corresponding control littermates (Figure 3D). Notably, the splenic viral load was significantly less in the *Il10*^{fl/fl}/CD11cCre^{pos} mice than in the *Il10*^{fl/fl}/CD11cCre^{neg} mice of the C57BL/6 as well as the BALB/c strains (Figure 3, C and D).

Taken together, T cell-derived Il10 suppresses the LCMV-induced immune response, as assessed by the LCMV-specific cytolytic activity of spleen cells. In the BALB/c mice, but not in the C57BL/6 mice, DC-derived Il10 contributes significantly to this effect. Nonetheless, T cell-specific deficiency as well as DC-specific deficiency in Il10 lead to reduced splenic viral loads in both the C57BL/6 and the BALB/c strains.

Figure 6 Course of the lymphocytic choriomeningitis virus (LCMV)-induced hepatitis in *Il10*^{-/-} mice and wild-type (wt) littermates. **A–D:** *Il10*^{-/-} mice and wt littermates of the C57BL/6 strain (**left panels**) or the BALB/c strain (**right panels**) were infected with i.p. injection of 10⁶ mouse-infectious units (IU) of LCMV strain WE. On the indicated days of infection, tissue samples and sera were prepared. **A:** Hepatitis activity was assessed in a blinded fashion according to Ishak scores on micrographs of hematoxylin and eosin-stained liver samples. **B and C:** Activity of aspartate aminotransferase (AST; **B**) and alanine aminotransferase (ALT; **C**) in serum samples prepared on the indicated days of infection. **D:** Viral load in the liver. Data are expressed as means ± SEM cumulative values from one experiment (**B** and **C**, C57BL/6 mice on day 2), two experiments (**A**, C57BL/6 mice on days 0, 2, and 4 and BALB/c mice; **B** and **C**, C57BL/6 mice on days 0 and 4 and BALB/c mice on days 2 and 4), three experiments (**A**, C57BL/6 mice on days 5, 6, and 10; **B** and **C**, C57BL/6 mice on day 5 and BALB/c mice on day 0), or four experiments (**A**, C57BL/6 mice on day 8; **B** and **C**, C57BL/6 and BALB/c mice on days 6, 8, and 10); or as means ± SEM cumulative log₁₀ values [in plaque-forming units (PFU) per gram] from two experiments (**D**, C57BL/6 mice on days 2, 4, 5, and 6 and BALB/c mice on days 2, 4, 5, 6, and 10), three experiments (**D**, BALB/c mice on day 8), four experiments (**D**, C57BL/6 mice on day 10), or five experiments (**D**, C57BL/6 mice on day 8). *n* = 1 (**B** and **C**, C57BL/6 mice per genotype on day 2); *n* = 2 (**A**, C57BL/6 mice per genotype on days 0 and 4 and BALB/c mice per genotype on days 0 and 10; **B** and **C**, C57BL/6 mice per genotype on day 4; **D**, BALB/c mice per genotype on days 5 and 10); *n* = 3 (**A**, C57BL/6 mice per genotype on day 2; **B** and **C**, BALB/c mice per genotype on day 4; **D**, C57BL/6 and BALB/c mice per genotype on days 2 and 4); *n* = 4 (**A**, C57BL/6 mice per genotype on day 6 and BALB/c mice per genotype on any day other than days 0 and 10; **B** and **C**, C57BL/6 mice per genotype on day 0 and BALB/c mice per genotype on day 5; **D**, C57BL/6 mice per genotype on days 5 and 6 and BALB/c mice per genotype on day 6); *n* = 5 (**A–C**, C57BL/6 mice per genotype on day 5; **B** and **C**, BALB/c mice per genotype on day 2); *n* = 7 (**A**, C57BL/6 mice per genotype on day 10; **D**, C57BL/6 *Il10*^{-/-} mice on day 10 and BALB/c mice per genotype on day 8); *n* = 8 (**B** and **C**, C57BL/6 mice per genotype on days 6, 8, and 10); *n* = 8 to 11 (**B** and **C**, BALB/c mice per genotype on days 0, 6, 8, and 10); *n* = 10 (**D**, C57BL/6 wt mice on day 10) *n* = 11 (**D**, C57BL/6 *Il10*^{-/-} mice on day 8); *n* = 12 (**D**, C57BL/6 wt mice on day 8); *n* = 13 (**A**, C57BL/6 mice per genotype on day 8). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (pair-wise comparisons using the two-sample *t*-test for independent samples).

Figure 7 Effects of *Il10* deficiency on the course of meningitis after intracerebral infection with lymphocytic choriomeningitis virus (LCMV). *Il10*^{-/-}, *Perf*^{-/-}, or *Il10*^{-/-}/*Perf*^{-/-} mice or wild-type (wt) littermates of the C57BL/6 (A and B) or BALB/c (C–F) strain were infected by intracerebral injection of 10³ mouse-infectious units (IU) of LCMV strain WE. Clinical appearance and survival of the mice were observed daily. Survival after infection is shown using Kaplan-Meier curves. **A** and **C**: *Il10*^{-/-} mice versus wt littermates of the C57BL/6 strain (A) and the BALB/c strain (C). Day-14 survival rates (95% CI): C57BL/6: *Il10*^{-/-}, 0 (0–0) versus wt, 17.6% (0–35.8); *P* = 0.930 (A); BALB/c: *Il10*^{-/-}, 28.6% (4.9–52.3) versus wt, 41.7% (13.8–69.5); *P* = 0.504 (C). **B** and **D**: *Il10*^{-/-}/*Perf*^{-/-} double-deficient mice versus *Perf*^{-/-} mice of the C57BL/6 strain (B) and the BALB/c strain (D). Day-21 survival rates (95% CI): C57BL/6: *Il10*^{-/-}/*Perf*^{-/-}, 24.0% (7.3–40.7) versus *Perf*^{-/-}, 66.7% (49.8–83.6) (B); BALB/c: *Il10*^{-/-}/*Perf*^{-/-}, 87.5% (77.3–97.7) versus *Perf*^{-/-}, 65.9% (52.0–79.8) (D). **E** and **F**: Serum concentrations of the proinflammatory cytokines interferon (Ifn)- γ (E) and tumor necrosis factor (Tnf) (F) in the BALB/c *Perf*^{-/-} and *Il10*^{-/-}/*Perf*^{-/-} mice shown in D. The area under the curve (AUC, weighted by the individual duration) was used as a summary measure. Data are expressed as cumulative values from three (A and C) or four (B and D) experiments, or as means $\pm$ SEM cumulative values from randomly chosen mice from two experiments (E and F). *n* = 4 (E and F, *Il10*^{-/-}/*Perf*^{-/-} mice on days $\geq$ 8); *n* = 8 (E and F, *Perf*^{-/-} mice on days $\geq$ 8); *n* = 10 (E and F, *Perf*^{-/-} mice on day 7); *n* = 11 (E and F, *Il10*^{-/-}/*Perf*^{-/-} mice on day 7); *n* = 12 (wt, C); *n* = 14 (*Il10*^{-/-}, C); *n* = 17 (wt, A); *n* = 22 (*Il10*^{-/-}, A); *n* = 25 (*Il10*^{-/-}/*Perf*^{-/-}, B); *n* = 30 (*Perf*^{-/-}, B); *n* = 40 (*Perf*^{-/-}, D); *n* = 44 (*Il10*^{-/-}/*Perf*^{-/-}, D). ****P* < 0.001, *****P* < 0.0001 [log-rank test (A–D); two-sample *t*-test (E and F)].

Il10 Dampens LCMV-Specific Immunopathology in Select Organs

Depending on the route of virus inoculation, the non-cytopathic LCMV induces various T cell-mediated immunopathological syndromes in acutely infected immunocompetent adult mice; for example, s.c. inoculation of LCMV into the hind footpad induces a DTH reaction,²⁶ i.p. injection leads to acute hepatitis,³⁶ and i.c. inoculation results in fatal choriomeningitis.³⁷ We addressed all of these manifestations for the immunoregulatory effects of *Il10*.

LCMV-Induced DTH Reaction

The typical course of the DTH reaction induced on intraplantar inoculation of LCMV²⁶ was similar between the *Il10*^{-/-} mice and the wt littermates of the C57BL/6 strain (Figure 4A) and the BALB/c strain (Figure 4B). The intensity of the DTH

reaction was enhanced with moderate significance in the *Il10*^{-/-} mice of both strains.

These data suggest that *Il10* contributes to regulating the DTH reaction on acute LCMV infection.

LCMV-Induced Acute Hepatitis

Acute hepatitis after i.p. injection of LCMV was monitored by both histopathology (Figures 5 and 6A) and by serum aminotransferase measurements (Figure 6, B and C). In contrast to the DTH reaction, *Il10* did not have an impact on the course of LCMV-induced acute hepatitis in the C57BL/6 or in the BALB/c mice. Nonetheless, the viral load in the liver was less in the C57BL/6 as well as in the BALB/c *Il10*^{-/-} mice compared with those in the respective wt littermates (Figure 6D).

LCMV-Induced Choriomeningitis

The kinetic properties and mortality rate of fatal choriomeningitis ensuing from i.c. LCMV infection were similar

Figure 8 Immunohistopathology of *Il10*^{-/-}/*Perf*^{-/-} double-deficient mice versus *Perf*^{-/-} mice after intracerebral infection with lymphocytic choriomeningitis virus (LCMV). BALB/c *Il10*^{-/-}/*Perf*^{-/-} (A, C, D, and G) mice and *Perf*^{-/-} littermates (B, D, F, and H) were intracerebrally infected with 10³ mouse-infectious units (IU) of LCMV strain WE. At day 15 of infection, mice were euthanized to prepare the brains for immunohistological analysis. Slides were stained with rat anti-mouse CD8 (A–D) or rat anti-mouse major histocompatibility complex (MHC) class II antigen (E–H) and were slightly counterstained with hemalum. A–D: Prominent infiltrates of CD8⁺ lymphocytes in the basal meninges, the fourth ventricle (asterisks), and perivascularly in a BALB/c *Il10*^{-/-}/*Perf*^{-/-} mouse (A and C) are shown; in contrast, fewer CD8⁺ T cells are present in a BALB/c *Perf*^{-/-} mouse (B and D). E–H: Microglial activation, as evidenced by up-regulation of MHC class II antigen, is much more pronounced in a BALB/c *Il10*^{-/-}/*Perf*^{-/-} mouse (E and G) compared with a BALB/c *Perf*^{-/-} mouse (F and H). Original magnification: ×200 (A, B, E, and F); ×400 (C, D, G, and H).

between the *Il10*^{-/-} mice and the wt littermates of both the C57BL/6 and BALB/c strains (Figure 7, A and C).

In contrast to this fulminant fatal immunopathology in immunocompetent mice, a subacute wasting disease develops in mice with impaired CD8⁺ T cell-mediated cytotoxicity, such as in *Perf*^{-/-} mice,¹⁶ with i.c. LCMV infection. The double-deficient *Il10*^{-/-}/*Perf*^{-/-} mice succumbed to i.c. LCMV infection significantly earlier and at greater rates than did the *Il10*-proficient/*Perf*^{-/-} control mice of the C57BL/6 as well as the BALB/c strains (Figure 7, B and D), once again indicating the immunoregulatory effect of *Il10*. However, the C57BL/6 and BALB/c mice showed major differences in the kinetics of LCMV-induced fatality. In the C57BL/6 mice, almost all of the

Il10^{-/-}/*Perf*^{-/-} mice as well as the *Perf*^{-/-} control mice died between days 5 and 15 of infection (Figure 7B), whereas in the BALB/c mice, about half of the *Il10*^{-/-}/*Perf*^{-/-} mice died between 15 and 40 days of infection (Figure 7D). Concurrent with this long-lasting course of disease, serum concentrations of Tnf and Ifnγ were significantly greater in the BALB/c *Il10*^{-/-}/*Perf*^{-/-} than in the *Perf*^{-/-} littermates during the entire course of infection (Figure 7, E and F). This finding is in accordance with the view that proinflammatory cytokines are involved in causing the LCMV-induced wasting disease in mice with compromised cytotoxic T-lymphocyte activity.³⁸

Brains of i.c. infected mice were histopathologically analyzed on day 15 of infection, that is, when no more fatalities were observed in the BALB/c *Perf*^{-/-} mice, whereas the BALB/c *Il10*^{-/-}/*Perf*^{-/-} mice were amid the fatal phase of meningitis. At that time, in mice of both genotypes, LCMV-induced basal meningitis and ventriculitis were obvious by histological examination (Figure 8, A and B). Severe meningitis and ventriculitis, dominated by CD8⁺ T cells (Figure 8A), with lymphocytes also having infiltrated the periventricular parenchyma (Figure 8C), had developed in the *Il10*^{-/-}/*Perf*^{-/-} mice (Table 1). In contrast, meningitis and ventriculitis were mild in the *Perf*^{-/-} littermates (Figure 8, B and D, and Table 1). Ubiquitous cerebral inflammation was also evidenced by the activation of microglia as detected by up-regulation of the major histocompatibility complex class II antigen in those cells. Similar to the lymphocytic infiltrates, microglia activation was more prominent in the *Il10*^{-/-}/*Perf*^{-/-} mice (Figure 8, E and G) than in the *Perf*^{-/-} littermates (Figure 8, F and H). By day 20 of infection, CNS inflammation in both genotypes regressed (data not shown). Nonetheless, meningeal, ventricular, and parenchymal CD8⁺ T cells were still more numerous and microglial activation was still more pronounced in the *Il10*^{-/-}/*Perf*^{-/-} mice than in the *Perf*^{-/-} mice.

Taken together, in i.c. LCMV-infected *Perf*^{-/-} mice, *Il10* deficiency markedly enhances both cerebral inflammation and systemic levels of proinflammatory cytokines.

In contrast to CNS inflammation, in the same mice shown in Figure 8, the severity of the concomitant hepatitis, ensuing from the systemic spreading of LCMV after i.c. inoculation, was similar between the BALB/c *Il10*^{-/-}/*Perf*^{-/-} mice

Table 1 Semiquantitative Evaluation of Inflammatory Infiltrates in the Meninges and the Ventricles on Day 15 of Intracerebral Infection with Lymphocytic Choriomeningitis Virus

Anatomical site	Mouse type	
	<i>Il10</i> ^{-/-} / <i>Perf</i> ^{-/-}	<i>Perf</i> ^{-/-}
Meninges	+++*	++
Ventricle	+++	+

n = 5 per genotype.

*0: no infiltrates.

+, single inflammatory cells; ++, small infiltrates of inflammatory cells, not exceeding one layer of lymphocytes; +++, large infiltrates of inflammatory cells, exceeding one layer of lymphocytes.

(means $\pm$ SEM Ishak score, 9.33 ± 0.47) and the *Perf*^{-/-} mice (9.0 ± 0.81) by day 15 of infection. These data confirm and extend our observations that Il10 does not affect the severity of LCMV-induced hepatitis (Figure 6A), regardless of whether CD8⁺ T cells are competent for perforin-mediated cytotoxicity. Furthermore, this finding indicates that, within the same mouse, the intensity of organ-specific immunopathology depends on both the activity of immune cells and the particular susceptibility of the respective organ.

Discussion

Persistent LCMV infection in mice is associated with impaired T-cell responses secondary to up-regulated Il10. Accordingly, blocking of Il10 action resulted in restored T-cell function and the elimination of the virus.^{9,10} Using the mouse model of acute LCMV infection, here we characterize the immunopathology resulting from T-cell responses in *Il10*^{-/-} mice.

Consistent with previous observations from several other groups using various LCMV strains,^{9,10} we report that LCMV strain WE infection was more effectively controlled in *Il10*^{-/-} than in wt mice during the first days of infection.

We now show that Il10 deficiency leads to enhanced immunopathology in select organs, indicating a price of the potential therapeutic use of blocking Il10 for the treatment of viral infections.

No Typical Th1 or Th2 Pattern in C57BL/6 or BALB/c Mice during Acute LCMV Infection

Most studies on LCMV-specific immune responses are performed in mice of the C57BL/6 strain. In various models of infection, this strain has been shown to be genetically disposed to generate a type 1-skewed cytokine milieu resulting in a particularly strong cellular CD8⁺ T cell-mediated immune response.¹²⁻¹⁴ The BALB/c strain has been shown to be the prototypic immunoregulatory antipode of the C57BL/6 strain because it is genetically disposed to generating a type 2-skewed cytokine pattern supportive of humoral rather than cell-mediated immune responses. Notably, Il10 belongs to the array of type 2 cytokines. During acute LCMV infection, systemic levels of Th1 and Th2 lead cytokines were not differentially skewed in C57BL/6 versus BALB/c mice according to a Th1- versus Th2-biased pattern. On the contrary, exceptionally high serum levels of the Th1 lead cytokine, Ifn γ , were detected in BALB/c mice during the early days of LCMV infection. This finding advises caution with regard to generalizing even widely accepted results to specific models.

CD4⁺ T Cells in Acute LCMV Infection: Helper versus Immunoregulatory Effects

Classic CD4⁺ T cells, functionally termed *T helper cells*, support the generation of CD8⁺ cytotoxic T cells in many viral infections, including LCMV infection.³⁹ But then CD4⁺ T cells are also known as major producers of the

immunoregulatory cytokine Il10, which is true also during—at least persistent—LCMV infection.^{9,10} This finding raised the question of whether and to which quantitative degree the complex helper effect of CD4⁺ T cells and the regulatory effect of the single cytokine Il10 together fine-tune the virus-specific activity of CD8⁺ T cells during acute LCMV infection. The enhanced virus-specific cytotoxic activity observed in CD8⁺ T cells from BALB/c *Il10*^{-/-} mice was repressed by mAb-mediated depletion of CD4⁺ T cells in BALB/c *Il10*^{-/-} mice to the level observed with CD8⁺ T cells from control-treated wt mice. The comparison of the outcomes of these two completely different treatment approaches, that is, genetic deletion of a single cytokine versus mAb-mediated depletion of a complete cell population, may appear as comparing apples to oranges. Nonetheless, the comparison of the effects of these different treatments on the same readout provides an impression of the regulatory power of Il10 on CD8⁺ T cells even during this very strong antiviral immune response.

Another important aspect of the Il10-mediated immunoregulatory circuitry is the cellular source of Il10, which may be secreted by many immunological cell types but also non-immunological cell types (reviewed by Moore et al¹ and O'Garra et al²). Using cell type-specific knockout mice, we revealed T lymphocytes as the most relevant source of Il10 dampening cytotoxic activity of CD8⁺ T cells in C57BL/6 as well as in BALB/c mice during acute LCMV infection. Additionally, DCs were identified as a source of Il10 dampening of LCMV-specific cytotoxic CD8⁺ T cells in BALB/c mice but not in C57BL/6 mice. Remarkably, DC-derived Il10 had a similar impact on the viral load in the spleen in BALB/c as well as in C57BL/6 mice. This discrepancy in viral concentrations between cytotoxicity assays performed *in vivo* and *in vitro* cautions against drawing from *in vitro* data far-reaching conclusions about the situation *in vivo*.

Of course, our identification of T cells and DCs as important sources of Il10 during acute LCMV infection does not preclude further cell types, such as myeloid-derived suppressor cells, as major producers of Il10 (reviewed by Goh et al⁴⁰), contributing to the total effect of Il10 to various degrees in different organs during acute LCMV infection.

LCMV-Induced Immunopathology

Enhanced cytotoxic T-cell responses may cause more adverse effects. We, therefore, performed systematic characterization of the consequences of Il10 deficiency on three defined immunopathological responses, that is, DTH in the footpad, hepatitis, and the eponymous choriomeningitis, induced by acute primary infection with the WE strain of LCMV via s.c., i.p., and i.c. injection, respectively.

Regarding the natural route of infection and the specific effects of different experimental routes of infection, LCMV infection requires specific considerations.

The most common natural infection of mice with LCMV is vertical transmission from persistently infected females to

embryos *in utero* or to their offspring during the first days after birth. *In utero* infection with this virus, which infects almost any murine cell type, occurs inevitably via the bloodstream. After birth, the pups are infected via shedding of LCMV at high concentrations in excrement and body fluids from their mothers. Fresh aerosols of body fluids from persistently LCMV-infected mice are the most common route of infection in humans also. Thus, the s.c., i.p., or i.c. route of virus inoculation are not used for mimicking natural infections. They are rather used as distinct models allowing *in vivo* and *ex vivo* experimentation in a highly reproducible setting. This way, LCMV immunology has contributed to revealing many important basic mechanisms of infection and immunity (reviewed by Zinkernagel⁴¹ and Oldstone⁴²).

Subcutaneous inoculation of LCMV into the hind footpad induces an immunopathologically mediated DTH reaction, that is, a transient footpad-swelling response, which is sequentially mediated by virus-induced CD8⁺ and CD4⁺ T cells.²⁶ *Il10*^{-/-} mice of both the C57BL/6 and the BALB/c strains developed a stronger swelling reaction than did the respective wt controls, suggesting, indeed, an adverse effect of Il10 deficiency on the development of DTH. The absolute increase of the swelling in *Il10*^{-/-} mice, although being statistically significant, may not appear impressive. It should be considered, however, that the skin of a mouse's paws is relatively tight and limited in its flexibility for volume increases of the paw. Therefore, the strong resistance of the skin against the swelling should be kept in mind when assessing the biological relevance of the limited increase in swelling compared with that in wt controls.

After i.p. injection, LCMV induces a fulminant but transient hepatitis in mice.³⁶ This immunopathology is consecutively mediated by the activity of natural killer cells, CD8⁺ T lymphocytes, and CD4⁺ T lymphocytes⁴³; this mediation does not require direct cell-to-cell contact between cytotoxic T lymphocytes and virus-infected hepatocytes.⁴⁴ However, histopathological analysis and serum aminotransferase measurements did not reveal any differences regardless of the strain of mice. Apparently, the vigorous T-cell response in the liver cannot be further intensified by the ablation of Il10.

The most severe LCMV-induced immunopathology is fatal choriomeningitis after i.c. inoculation of the virus.³⁷ The fulminant fatality among the majority of both *Il10*^{-/-} and wt mice in a narrow timeframe prevented direct analysis of underlying immunoregulatory mechanisms. However, the course of the disease can be slowed by partial impairment of the cytolytic activity of CD8⁺ T cells. The prototypic example is *Perf*^{-/-} mice. In *Perf*^{-/-} mice infected with LCMV i.c., T lymphocytes are continually stimulated by the high viral load to secrete large quantities of proinflammatory cytokines, leading to a protracted wasting syndrome.^{16,38} The majority of *Il10*^{-/-}/*Perf*^{-/-} mice of both the C57BL/6 and the BALB/c strains succumbed after i.c. inoculation of LCMV, whereas a significantly smaller percentage of the Il10-proficient *Perf*^{-/-} controls died. Specifically, strongly increased lymphatic infiltrates and more pronounced

inflammatory activation of microglia within the brain were accompanied by significantly elevated serum levels of proinflammatory cytokines, which contribute to the fatal LCMV-induced wasting disease.³⁸ Thus, local and systemic parameters were indicative of the cellular mechanisms that increase the risk for mortality in *Il10*^{-/-} mice infected with LCMV i.c.

Collectively, the strongly increased activity of LCMV-specific CD8⁺ T cells in Il10-deficient mice was accompanied by increased LCMV-related immunopathology. Notably, a differential impact of Il10 on LCMV-induced immunopathology was observed in various nonlymphatic target organs. Clearly, the therapeutic manipulation of the immunosuppressive master regulator IL10 requires further in-depth characterization of possible adverse effects.

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