Mechanisms of control of acute Friend virus infection by CD4⁺ T helper cells and their functional impairment by regulatory T cells

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The role of cytotoxic CD8⁺ T cells is well defined in retroviral immunity but the role of CD4⁺ T helper (Th) cells is poorly understood. The Friend retrovirus (FV) murine infection model is a good model to study immune responses in retroviral infections and hence was used to characterize the role of Th cells during acute infection. In vivo depletion of Th cells in acutely infected mice demonstrated that Th cells were vital in controlling viral spread and onset of erythroleukaemia and for the maintenance of FV-specific CD8⁺ T-cell and neutralizing antibody responses. Kinetic analysis of FV-specific Th-cell responses using class-II tetramers showed that the magnitude of the Th-cell response correlated with the level of resistance to FV-induced leukaemia in different mouse strains. FV-specific CD4⁺ T-cell receptor β-transgenic (TCRβ-tg) T cells were adoptively transferred into mice infected for different time periods [1, 2 and 3 weeks post-infection (p.i.)] to investigate the direct antiviral effect of CD4⁺ T cells in FV infection. Results indicated that FV-specific CD4⁺ TCRβ-tg T cells were functionally active until 2 weeks p.i., retaining their ability to produce gamma interferon (IFN-γ) and reduce viral loads. However, the donor cells lost their antiviral activity starting from 3 weeks p.i. Interestingly, in vivo depletion of regulatory T cells (Tregs) at this time point restored IFN-γ production by transferred CD4⁺ T cells. The current study reveals that Th cells were critical for recovery from acute FV infection but were functionally impaired during the late phase of acute infection due to induced Tregs.

INTRODUCTION

Cytotoxic CD8⁺ T cells (CD8⁺ CTLs) and CD4⁺ T helper (Th) cells are crucial for all adaptive immune responses during ongoing infections. CD8⁺ CTLs kill target cells infected with intracellular pathogens, thereby removing sites of pathogen replication. Th cells are a subpopulation of CD4⁺ T cells which are specialized in providing supporting signals to other cells such as B cells and CD8⁺ T cells through either secretion of cytokines or direct cell-to-cell interactions. Th cells provide help to a number of key immune responses involved in controlling viruses, for example in lymphocytic choriomeningitis virus (LCMV) (Fayolle et al., 1991), influenza virus (Graham et al., 1994), vesicular stomatitis virus (Maloy et al., 1999), polio virus (Mahon et al., 1995) and murine γ herpes virus (Cardin et al., 1996) infections. The crucial importance of Th cells in viral immunity is vividly demonstrated in human immunodeficiency virus type 1 (HIV-1) infection, where Th cells are the main targets and are depleted by the virus. Absence of Th cells strongly affects the virus-specific cytotoxic T cell (CTL) (Altfeld & Rosenberg, 2000; Spiegel et al., 2000) and neutralizing antibody (Poignard et al., 1999) responses, leading to AIDS. Furthermore, Th cells have been shown to induce direct antiviral effects via secretion of cytokines, such as gamma interferon (IFN-γ) and tumour necrosis factors (TNF) (Gagnon et al., 1999), in murine infections of LCMV (Varga & Welsh, 1998), herpes simplex virus (HSV) (Manickan et al., 1995), vaccinia virus (VV) (Maloy et al., 2000) and measles virus (Reich et al., 1992). IFN-γ-producing Th cells are also present in HIV-1 infection (Littaua et al., 1992; Norris et al., 2001; Sethi et al., 1988). Moreover, C-C chemokines secreted by Th cells suppress infection by chemokine receptor-5-dependent HIV-1 viruses (Furci et al., 1997). However, most of the findings on mechanisms of Th-cell-mediated protection in HIV-1 have been obtained during the chronic stages of infection. This is because primary HIV-1 infection is asymptomatic, so very few patients are
available for immunological studies. HIV vaccine research would highly benefit from additional knowledge about the initial interaction of retroviruses with CD4+ Th cells during acute infection.

Mouse models of HIV infection are not available and, as a result, the Friend retrovirus (FV) model has been used extensively to study the mechanisms of immunity against retroviruses (Hasenkrug & Chesebro, 1997; Hasenkrug & Dittmer, 2000). FV is a retroviral complex that induces lethal erythroleukaemia in susceptible strains of mice (Dittmer & Hasenkrug, 2001; Hasenkrug & Dittmer, 2000). However, mice that mount efficient immune responses against the virus due to their major histocompatibility complex (MHC) background are resistant to leukaemia (Chesebro et al., 1990). Recovery from acute infection is dependent upon cytotoxic killing of FV-infected cells by CD8+ CTLs (Zelinskyy et al., 2004). On the other hand, Th cells are known to be critical in controlling FV replication and spread in persistently infected mice (Hasenkrug et al., 1998a). Moreover, vaccination studies suggested the crucial importance of Th cells, CTLs and B cells for the induction of complete protection against FV infection (Dittmer et al., 1999). Also, CD4+ T cells were required for recovery in at least 50% of the resistant mice infected with FV (Robertson et al., 1992). However, in that study, effector functions of Th cells were not investigated and, moreover, there were no tools available for quantification of Th-cell responses. Furthermore, experiments were performed with a contaminated FV stock containing lactate dehydrogenase virus (LDV), which can delay immune responses against FV (Robertson et al., 2007). Therefore, we investigated the role of Th cells during acute FV infection using an LDV-free FV stock and also characterized the effector functions of Th cells. Here, we show that Th cells played a cardinal role in limiting the pathological consequences during acute FV infection by mediating help to CD8+ T cells and B cells. Furthermore, the magnitude of FV-specific effector Th-cell responses was found to be dependent on the MHC background of infected mice.

In spite of an effective CD8+ T-cell immune response during an acute FV infection, high recovery mice are never able to clear the virus completely, and remain persistently infected for life (Dittmer & Hasenkrug, 2001; Hasenkrug & Dittmer, 2000). During bacterial, parasitic and viral infections, immunosuppressive regulatory T cells (Tregs) expand, which dampens T-cell responses (Robertson & Hasenkrug, 2006). In some viral infections, the immune response is more detrimental than the virus itself, but immunosuppression by Tregs can prevent pathology in the host. However, immunosuppressive effects on T cells can also be beneficial to the virus, by prolonging recovery of the infected host, leading to persistent infections. Functional impairment of FV-specific CD8+ T cells in persistent FV infections is mediated by Tregs (Dittmer et al., 2004; Zelinskyy et al., 2006). Importantly, these Tregs expand 2–3 weeks after acute FV infection and consequently suppress the functions of effector CD8+ T cells. In addition, Antunes et al. (2008) showed that bone marrow pathology induced by FV-specific Th cells in FV-infected RAG1-/- mice can be prevented by exogenous Tregs. However, in this case the role of Tregs was only studied in lymphopenic hosts, where it is known that suppression by Tregs is accentuated. What is not known is whether Tregs can modulate antiviral Th-cell responses during acute FV infection in immunocompetent wild-type mice. Therefore, we adoptively transferred FV-specific CD4+ T-cell receptor β-transgenic (TCRβ-tg) T cells into FV-infected mice and performed a kinetic analysis of their antiviral responses during acute FV infection. Results indicated that FV-specific CD4+ T cells with effector phenotype were functionally impaired at 3 weeks post-infection (p.i.), resulting in the loss of antiviral IFN-γ. Depletion of Tregs led to a significant increase in the frequency of IFN-γ-producing FV-specific Th cells. These results suggest that Tregs induce immunosuppression of Th-cell immunity during acute FV infection and, thereby, may contribute towards viral persistence.

RESULTS

Th cells are important in controlling viral replication and preventing erythroleukaemia during acute FV infection

Th cells are important in vaccine-induced protection against FV infections (Dittmer & Hasenkrug, 2000) and in restricting FV replication and spread in persistently infected mice (Hasenkrug et al., 1998a). Additionally, deficiency of CD4+ T cells resulted in leukaemia in almost 50% of FV-resistant animals during acute FV infection (Robertson et al., 1992). However, the mechanisms underlying the influence of CD4+ T cells on recovery from FV-induced disease remain elusive. To better understand the importance of CD4+ T cells in recovery from FV infection, mice were injected with anti-CD4 monoclonal antibodies to deplete CD4+ T cells, while control animals received PBS. The effectiveness of CD4 depletion was greater than 98%. After 1 week, CD4-depleted mice and non-depleted controls were infected with FV. Spleens from these mice were palpated at weekly intervals to monitor splenomegaly (Hasenkrug et al., 1998a). CD4-depleted mice had much enlarged spleens compared with non-depleted mice at 4 weeks p.i. This time point was chosen to determine how CD4 T-cell depletions affected FV-induced erythroleukaemia and spleen viral loads. CD4+ T-cell numbers were still reduced in depleted mice, indicating that the effect of depletion lasts for at least 4 weeks. The mean spleen weight of CD4-depleted mice was 2.6 g at this time point (Fig. 1a). This was in sharp contrast with the spleen weights of non-depleted mice, which had a mean spleen weight of 0.21 g, indicating that FV-induced disease had resolved by that time. These results suggest that the absence of CD4+ T cells affects the normal
recovery from FV-induced disease and results in gross splenomegaly. Infectious centres (IC) assays were performed to determine whether the depletion of CD4+ T cells affected levels of viral replication in the spleen. The assay revealed that there were significantly more ICs in CD4-depleted mice (mean = 1.375 × 10^6) than in non-depleted mice (mean = 2.376 × 10^5) (Fig. 1b). Thus, the depletion of the CD4+ T-cell population resulted in increased FV replication during the recovery phase of FV infection (4 weeks p.i.) and progression to FV-induced erythroleukaemia in the resistant mouse strain (C57BL/10 × A.BY) F1.

**CD4+ T-cell help is essential for the maintenance of FV-specific effector and memory CD8+ T-cell responses and for efficient antibody responses during acute FV infection**

We have established that CD4 depletions resulted in the loss of immunity to FV infection in resistant mice. The mechanisms by which CD4+ T cells mediated protection against FV were of relevance to our study. CD4+ Th cells coordinate various immune responses involving B cells and CTLs. There has been contrasting evidence related to the requirement of help from CD4+ T cells for the activation, expansion and survival of CD8+ T cells in various infections. Thus, it was of interest to investigate the role of CD4+ T-cell-mediated help to CD8+ T cells in FV infection. We quantified the population of FV-specific effector CD8+ T cells (CD8+CD43+Tet+ T cells) by staining the splenocytes with FV-specific DΔgagL MHC class-I tetramers. At 4 weeks p.i., there was a remarkable decrease in the population of specific effector CD8+ T cells in spleens of CD4-depleted mice compared with non-depleted mice (Fig. 2a). Th cells are also known to be important for the maintenance of the long-lived memory CD8+ T-cell pool (Buller et al., 1987; Janssen et al., 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003). Kaech et al. (2003) defined memory CD8+ T cells as the population with expression of CD127 on virus-specific CD8+ T cells (Tetramer+). Here, absolute cell numbers of FV-specific memory CD8+ T cells (CD8+Tet+CD127+ T cells) were significantly lower in CD4-depleted mice compared with non-depleted mice (Fig. 2b). It has been described previously that, at 7 and 14 days p.i., FV-specific effector CD8+ T-cell responses in spleens were not affected by CD4+ T-cell depletions (Robertson et al., 2007). Thus, it is apparent that early recovery from FV infections mediated by CD8+ T-cell responses is CD4+ T-cell-independent, but long-term maintenance of memory CD8+ T-cell responses requires help from CD4+ T cells.

CD4+ T-cell help is indispensable for the production of neutralizing antibodies in various infections. Furthermore, virus-neutralizing antibodies are essential for recovery from FV infection, as concluded from vaccine studies. Hence, to determine whether B cells require help from Th cells for secretion of antibodies, plasma samples from CD4-depleted and non-depleted mice were tested for FV-specific antibody levels. At 4 weeks p.i., the mean Friend murine helper virus (F-MuLV)-specific binding antibody titre and the neutralizing titre for FV-infected non-depleted mice were significantly higher compared with the mean titres of CD4-depleted FV-infected mice (Fig. 2c). Thus, Th cells were critical for aiding B cells to induce virus-specific antibodies. In vivo depletion of Th cells in acute FV infection demonstrated that Th cells were critical for the maintenance of CD8 and neutralizing antibody responses.

**Resistance to FV-induced disease correlates with the magnitude of H-2A-restricted CD4+ T-cell responses**

It is known that recovery from FV-induced disease depends upon an array of genes mapped to the MHC H-2 region of mice, of which H-2A can influence the effectiveness of Th-cell responses (Chesebro et al., 1990). Additionally, immunization with a single Th-cell epitope in the gp70 envelope (Env) glycoprotein of F-MuLV induced potent Aa-restricted Th-cell responses that conferred partial
immunity to FV infection (Iwashiro et al., 2001b; Miyazawa et al., 1995). Hence, for the current study, we focused on this epitope and investigated how the H-2A\textsuperscript{b} background of a mouse controls the protective T-cell response during acute FV infection. For this purpose, we quantified the population of FV-specific effector CD4\textsuperscript{+} T cells during acute FV infection. Acute FV infection was established in (C57BL/10 × A.BY) F\textsubscript{1} mice and depletion of CD4\textsuperscript{+} T cells was performed as described in Methods. Spleen cells from CD4-depleted and non-depleted mice were isolated at 4 weeks p.i. (a, b) Percentage of FV-specific effector CD8\textsuperscript{+} T cells (a) and absolute cell numbers of memory CD8\textsuperscript{+} T cells (b) determined in non-depleted FV-infected and CD4-depleted FV-infected mice. Gated live CD8\textsuperscript{+} T cells were co-stained for the activation-associated glycoform of CD43 and FV-specific D\textsuperscript{b} gagL MHC class-I tetramer (a) and for FV-specific D\textsuperscript{b} gagL MHC class-I tetramer and CD127 to detect virus-specific memory CD8\textsuperscript{+} T cells (b). (a, b) Each dot represents an individual mouse and mean values are indicated by a line. (c, d) Mean neutralizing antibody titres (c) and total binding antibody titres (d) (represented as log\textsubscript{2} geometric means) of frozen plasma samples from naïve, CD4-depleted and non-depleted mice. Error bars represent SEM. (a–d) \( P \) values were determined by the Mann–Whitney U test. The experiments were repeated twice, with comparable results.

This kinetic study showed that the peak of the FV-specific effector T-cell response was at 10 days p.i. (Fig. 3a). Interestingly, there was a significant difference in peak CD4\textsuperscript{+}Tet\textsuperscript{+} T-cell responses, with high recovery mice having 58% more virus-specific CD4\textsuperscript{+} T cells than intermediate recovery mice. In addition, the percentage of total CD4\textsuperscript{+} T cells expressing the effector cell markers CD43 and CD69 was also higher in high-recovery mice than intermediate recovery mice at 10 days p.i. (Fig. 3b). Therefore, we observed a significant difference in overall activation of effector T cells and FV-specific T-cell responses between high and intermediate recovery mice. Thus, the resistance of mice to FV-induced disease correlated with the magnitude of the H-2A-restricted CD4\textsuperscript{+} T-cell response. These data support findings from the FV vaccination experiment which demonstrated that the H-2A\textsuperscript{b/b} genotype influenced CD4\textsuperscript{+} T-cell-mediated immune responses (Chesebro et al., 1990; Miyazawa et al., 1988). From these studies, we knew that resistance to FV infection was dependent on the MHC background. However, for the first time, the population of H-2A\textsuperscript{b}-restricted FV-specific CD4\textsuperscript{+} T cells in mice with a different recovery status could be accurately quantified using class-II tetramers. Thus, our study suggests that the differential resistance to FV infection in mice expressing different levels of H-2A\textsuperscript{b} was at least in part due to the difference in the magnitude of CD4\textsuperscript{+} T-cell responses.

FV-specific CD4\textsuperscript{+} TCR\(\beta\)-tg T cells produce IFN-\(\gamma\) and reduce viral loads in FV-infected mice

Using adoptively transferred FV-specific CD8\textsuperscript{+} T cells from CD8\textsuperscript{+} T-cell receptor transgenic mice, it was shown that such cells dramatically reduce viral loads during acute FV infection, but fail to reduce viral loads in persistently infected mice (Dittmer et al., 2004). In the current study, we address the antiviral activity of CD4\textsuperscript{+} T cells during different phases of FV infection. We made use of CD4\textsuperscript{+} TCR\(\beta\)-tg mice transgenically expressing the TCR\(\beta\) chain (Antunes et al., 2008) that recognizes the FV-specific CD4\textsuperscript{+} T-cell epitope H19-Env. In principle, these FV-specific T cells have been shown to reduce FV loads in vivo (Antunes et al., 2008).

Donor CD4\textsuperscript{+} T cells from transgenic mice were differentiated from the endogenous host T cells of recipient mice on the basis of congenic markers. T cells from donor mice express CD45.1, while T cells from the recipients express CD45.2. For the present study, only antigen-inexperienced T cells were used. So, the donor cells are naive and the recipient T cells are antigen-experienced. Using adoptively transferred FV-specific CD8\textsuperscript{+} T cells from CD8\textsuperscript{+} T-cell receptor transgenic mice, it was shown that such cells dramatically reduce viral loads during acute FV infection, but fail to reduce viral loads in persistently infected mice (Dittmer et al., 2004). In the current study, we address the antiviral activity of CD4\textsuperscript{+} T cells during different phases of FV infection. We made use of CD4\textsuperscript{+} TCR\(\beta\)-tg mice transgenically expressing the TCR\(\beta\) chain (Antunes et al., 2008) that recognizes the FV-specific CD4\textsuperscript{+} T-cell epitope H19-Env. In principle, these FV-specific T cells have been shown to reduce FV loads in vivo (Antunes et al., 2008).

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Intracellular cytokine staining for IFN-γ was significantly elevated compared with donor naive mice by flow cytometry. Adoptive transfer of FV-specific TCRβ-tg CD4+ T cells were utilized for the adoptive transfer experiment. One week after adoptive transfer of CD4+ TCRβ-tg CD4+ T cells into FV-infected recipient mice, CD4+ T cells were reisolated and analysed by flow cytometry. Adoptive transfer of FV-specific TCRβ-tg CD4+ T cells into naive control mice indicated that only poor expansion of the donor cells occurs when no specific antigen is present. However, a strong expansion of FV-specific TCRβ-tg CD4+ T cells was observed after transfer into mice infected with FV for 1, 2 or 3 weeks; representative data are shown in Fig. 4(a). Furthermore, expansion of the donor cells was paralleled by an activation of the cells after recognizing their cognate antigen. Whereas very few cells that were recovered from naïve mice displayed an effector phenotype (CD43low and CD62Llow), the numbers of effector CD4+ T cells reisolated from animals infected with FV for 1 week post-transfer. The highest percentages of CD4+ TCRβ-tg T cells with effector phenotype were reisolated from animals infected with FV for 1 week (Fig. 4b). Nevertheless, it was also observed that CD4+ TCRβ-tg T cells transferred into mice infected with FV for 3 weeks significantly activated. Given the rapid expansion and activation of the FV-specific CD8+ T cells, which were unable to produce IFN-γ and cytolyltic molecules following adoptive transfer into persistently infected mice even though they had an effector phenotype (Dittmer et al., 2004; Zelinskyy et al., 2005). To probe further whether transferred FV-specific CD4+ TCRβ-tg T cells with effector phenotype were producing IFN-γ, we performed a co-staining experiment for intracellular IFN-γ production and CD43 expression. A significant number of donor CD4+ T cells reisolated from recipient mice infected for 1 or 2 weeks had an effector phenotype and produced IFN-γ. However, in spite of sufficient activation, donor CD4+ T cells reisolated from mice infected with FV for 3 weeks did not significantly produce IFN-γ (Fig. 4d).

Fig. 3. Kinetics of FV-specific effector CD4+ T-cell responses. High recovery (H-2A b/b) (C57BL/10 x A.BY) F1 mice and intermediate recovery (H-2A b/k) (C57BL/10.A x A.BY) F1 mice were acutely infected with FV. (a) Mean percentages ± SEM of virus-specific CD4+ T cells reactive with I-A b MHC class-II tetramers specific for FV-Env epitope (H-19 Env) in H-2A b/b (•) and H-2A b/k (■) mice at various time points of infection (n=7–10 mice per group). (b) Mean percentages ± SEM of live CD4+ T cells in the spleen expressing cell-surface markers CD69 and CD43 at 10 days p.i. in H-2A b/b (black bars) and H-2A b/k (white bars) mice (n=6–10 mice per group). P values were determined by the Mann–Whitney U test. The experiment was repeated three times, with comparable results.
Fig. 4. Antiviral activity of adoptively transferred CD4⁺ TCRβ-tg T cells. High recovery (H-2Aβ/b) (C57BL/10 × A.BY) F₁ mice expressing CD45.2 were acutely infected with FV for either 1, 2 or 3 weeks. CD4⁺ TCRβ-tg cells (7.5 × 10⁶) from mice expressing CD45.1 were transferred into naïve and FV-infected recipient mice. One week after transfer, CD4⁺ T cells from spleens of recipient mice were reisolated and investigated. (a) Representative data of expansion of donor CD4⁺ T cells (CD45.1⁺ T cells) reisolated from naïve, 1-, 2- and 3-week-FV-infected recipient mice. Percentages of positive cells (upper right quadrant) are given in the key above each dot blot. (b) Activation of donor CD4⁺ T cells reisolated from naïve, 1-, 2- and 3-week-FV-infected recipient mice investigated by staining spleen cells for CD43 and CD62L surface markers. Mean percentages ± SEM of CD4⁺ T cells expressing CD43(high) (●) or CD62L(low) (■) in 1-, 2- and 3-week-FV-infected recipient mice were statistically different (P<0.05) from naïve recipient mice. P values were determined by the Mann–Whitney U test (n=7 mice per group). (c) Percentage of donor CD4⁺ T cells producing IFN-γ after transfer into naïve and FV-infected recipient mice. Each dot represents an individual mouse and the mean percentages are indicated by a line. P values were determined by the Dunnett’s multiple comparison test. (d) Donor CD4⁺ T cells co-expressing IFN-γ and CD43 gated on donor (CD45.1) CD4⁺ T cells. Mean percentages ± SEM of activated FV-specific CD4⁺ TCRβ⁻/tg T cells producing IFN-γ; white bars represent the percentages of activated FV-specific CD4⁺ TCRβ⁻/tg T cells. Mean percentages ± SEM of respective populations are shown. (e) Viral loads in 1-, 2- and 3-week-FV-infected recipient mice calculated by counting the number of ICs per spleen. Each dot represents an individual mouse and the mean viral loads are indicated by a line. P values for the difference in means (log_{10}) were determined by the Mann–Whitney U test. All experiments were repeated three times, with comparable results.
did not have the capacity to reduce viral loads in vivo (Fig. 4e).

These results indicated that the CD4+ TCRβ-tg T cells were effective at reducing viral loads in mice infected with FV for 1 or 2 weeks but were ineffective at reducing viral loads in mice infected with FV for 3 weeks. Thus, although the FV-specific CD4+ T cells expanded and upregulated the activation marker CD43 following adoptive transfer into infected mice, the cells were functionally impaired in mice infected with FV for 3 weeks (Fig. 4d), resulting in loss of their antiviral activity.

Suppression of FV-specific CD4+ T-cell responses by regulatory T cells

We previously reported that the functional impairment of FV-specific CD8+ T cells during chronic infection is mediated by CD4+ Foxp3+ Tregs, which expand during the late phase of acute FV infection (2–3 weeks p.i.) (Dittmer et al., 2004; Zelinskyy et al., 2006). In the current study, we speculated that the low frequency of IFN-γ-producing FV-specific CD4+ T cells at 3 weeks p.i. could also be a result of Treg-mediated CD4+ T-cell dysfunction. To determine whether Tregs were inhibiting the antiviral functions of virus-specific CD4+ Th cells, we used a mouse model in which Foxp3-expressing Tregs can be experimentally depleted with diphtheria toxin (DT) treatment.

Fig. 5. Suppression of FV-specific CD4+ T-cell responses by Tregs. (a, b) High recovery (H-2A b/b) DEREG.Y10 mice expressing CD45.2 were acutely infected with FV for 3 weeks. CD4+ TCRβ-tg T cells (7.5 × 10^6) from mice expressing CD45.1 were transferred into FV-infected recipient mice. Simultaneously, mice also received DT on alternate days for 1 week. At 7 days post-transfer, IFN-γ production in (a) CD4+ TCRβ-tg T cells reisolated from FV-infected mice and (b) endogenous (CD45.2+) CD4+ T cells of the host were assayed. Each dot represents an individual mouse and the mean percentages are indicated by a line. P values were determined by the Mann–Whitney U test. Treatment of non-DEREG mice with DT did not influence T-cell responses (data not shown). (c) Viral loads in FV-infected Treg-depleted (+CD4+ DT) and non-depleted DEREG.Y10 (+CD4+ mouse that received CD4+ TCRβ-tg T cells (n=7 per group). DEREG.Y10 mice that did not receive any DT or transferred cells were used as controls. All experiments were repeated twice, with comparable results.
transferred CD4\(^+\) TCR\(\beta\)-tg T cells (Fig. 5a). Hence, it is clearly evident that Tregs play a crucial antiviral role in suppressing immune functions of Th cells in the late phase of acute FV infection.

**DISCUSSION**

Th cells represent a critical and integral part of a functional immune system and are well characterized in many viral infections, but their antiviral role in retroviral immunity is poorly understood. The focus of this study was to elucidate the role of Th cells during acute FV infection. In vivo depletion of CD4\(^+\) T cells underscored the vital role of Th cells in controlling viral spread and the onset of FV-induced erythroleukaeemia. It has been shown previously that recovery from acute FV infection requires strong effector responses elicited by CD8\(^+\) CTLs that control viral replication (Zelinsky et al., 2004). On the other hand, restriction of persistent FV infection is CD8\(^+\) T-cell-independent and solely dependent upon CD4\(^+\) T-cell effector responses (Hasenkrug et al., 1998a). Our results reveal that, in spite of effective CD8\(^+\) T-cell responses during acute FV infections, the absence of CD4\(^+\) T cells in high recovery mice rendered them susceptible to FV-induced disease. In this respect, CD4\(^+\) T cells are substantially crucial for controlling FV replication during acute infection.

In the current study, we explored the mechanisms of Th-cell-mediated immunity during acute FV infections. Numerous studies have defined the role of CD4\(^+\) T cells in providing ‘help’ to key immune functions of CD8\(^+\) T cells and B cells involved in antiviral immunity. Our results clearly support this concept for retroviral infections. However, the requirement of CD4\(^+\) T cells in helping CD8\(^+\) T-cell responses against pathogens remains a controversial field. It has been well documented for several murine infections that CD4\(^+\) T-cell help is not needed during the priming phase of CD8\(^+\) T-cell responses (Buller et al., 1987; Janssen et al., 2003; Novy et al., 2007; Shedlock & Shen, 2003; Sun & Bevan, 2003). This lack of requirement for CD4\(^+\) T cells during the programming phase of infection is probably because most pathogens elicit strong inflammatory stimuli that directly activate antigen-presenting cells (Buller et al., 1987; Wu & Liu, 1994). This is in agreement with our previous work which showed that the absence of CD4\(^+\) T cells during the early phase (up to 2 weeks) of FV infection did not influence FV-specific CD8\(^+\) T-cell responses in FV-infected mice (Robertson et al., 2007). In contrast, primary CD8\(^+\) T-cell responses to other pathogens are dependent on help from CD4\(^+\) T cells (Marzo et al., 2004; Ribberd et al., 2000; Smith et al., 2004; Yang et al., 1995). Interestingly, we demonstrate here that, during the late phase of acute infection (4 weeks p.i.), deficiency of CD4\(^+\) T cells in FV-infected mice affected the maintenance of FV-specific effector CD8\(^+\) T-cell responses. In HIV-1 infection, the ability of virus-specific CD8\(^+\) T cells to secrete IFN-\(\gamma\) is compromised due to lack of help from CD4\(^+\) T cells (Spiegel et al., 2000). In other infection models, Th cells were also required during the maintenance phase of the long-lived memory CD8\(^+\) T-cell pool (Buller et al., 1987; Janssen et al., 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003). CD4\(^+\) T-cell help provided during the primary response against infections such as those caused by LCMV, VV and *Listeria monocytogenes* delivers the necessary instructive signals for the generation of fully functional memory CD8\(^+\) T cells (Janssen et al., 2003; Shedlock & Shen, 2003). In line with this theory, we provide evidence that, during the late acute phase of FV infection, mice lacking CD4\(^+\) T cells had poor expansion of FV-specific CD8\(^+\) T cells expressing the memory cell marker CD127.

Thus, Th cells seem to be critical for the induction of retrovirus-specific CD8\(^+\) memory T-cell responses. This finding might be of interest for the development of retroviral vaccines.

Induction of antiviral neutralizing antibodies in conjunction with cellular immune responses is an important mode of defence against most viruses. Lack of HIV-1-specific Th cells in established HIV-1 infection hampers the host’s ability to generate effective antibody responses (Pantaleo & Walker, 2001). In FV infection too, spontaneous recovery from FV requires the induction of virus-neutralizing antibodies (Chesebro & Wehrly, 1979). In the present study, the lack of help from CD4\(^+\) T cells resulted in significant loss of FV-specific neutralizing antibodies during acute FV infection. However, we cannot rule out that low antibody titres in CD4-depleted mice may have been partially influenced by high viral loads, as antibodies may have been bound to viral particles. Therefore, our results suggest that CD4\(^+\) T cells are important for the production of FV-specific antibodies in acute retroviral infection. Besides, vaccine studies emphasize the importance of virus-neutralizing antibodies along with the priming of T cells for sterilizing immunity against FV (Dittmer et al., 1999). Thus, it is apparent that Th cells contribute towards B-cell and CD8\(^+\) T-cell responses, thereby influencing recovery from FV-induced disease during acute infection.

The recovery of mice from FV-induced disease depends upon their H-2A genotype, which influences the FV-specific CD4\(^+\) T-cell response against FV (Chesebro et al., 1990). However, how the H-2A genotype influences the quantity of the CD4\(^+\) T-cell responses against FV had not been investigated. Here, we used MHC class-II tetramers for the first time to compare the kinetics of CD4\(^+\) T-cell responses against FV in high and intermediate recovery mice. Our study extends earlier results to show that resistance to FV-induced disease correlated with the magnitude of H-2A\(^b\)-restricted CD4\(^+\) T-cell responses (Chesebro et al., 1990; Miyazawa et al., 1995). This might be because high recovery mice may be able to present more CD4 epitopes due to the 100 % H-2Ab allele frequency than low antibody titres in CD4-depleted mice. Therefore, our results suggest that CD4\(^+\) T cells had poor expansion of FV-specific CD8\(^+\) T cells expressing the memory cell marker CD127. In this respect, CD4\(^+\) T cells are substantially crucial for controlling FV replication during acute infection.

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responses in intermediate recovery mice cannot be measured, due to the lack of specific tools. For humans, variations at immune-gene loci determine the clinical course and outcome of HIV-1, hepatitis C virus and hepatitis B virus (HBV) infections (Martin & Carrington, 2005). Thus, strategies for vaccine development need to consider the genetic diversity of immune response genes that can influence resistance or susceptibility to several different diseases.

Apart from being the ‘helper’ arm of the immune system, CD4\(^+\) T cells can have direct antiviral activities. CD4\(^+\) T cells have been described to induce direct anti-leukaemic effects in FV-infected mice progressing to erythroleukaemia (Pourbohloul et al., 1992). One mechanism of antiviral activity of CD4\(^+\) T cells is the production of cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\) (Gagnon et al., 1999). IFN-\(\gamma\) production by CD4\(^+\) T cells is a key component in the control of persistent FV infection in mice (Stromnes et al., 2002). In the current study, we investigated the direct antiviral effect of FV-specific Th cells using CD4\(^+\) TCR\(\beta\)-tg cells specific for the F-MuLV Env epitope. Transfer of such cells into acutely infected mice did not affect secondary immune responses like CD8\(^+\) T-cell and B-cell responses (data not shown). This suggests that the antiviral immunity detected after transfer of CD4\(^+\) TCR\(\beta\)-tg T cells was due to the direct effect of these cells. Adoptively transferred FV-specific CD4\(^+\) TCR\(\beta\)-tg T cells rapidly expanded and became activated in acutely infected mice. Donor CD4\(^+\) T cells displayed IFN-\(\gamma\) responses until 2 weeks p.i. This correlated with a significant reduction in viral loads in recipient mice. IFN-\(\gamma\)-producing CD4\(^+\) T cells were also observed in the HBV transgenic mouse model (Franco et al., 1997). Additionally, IFN-\(\gamma\) production by HIV-1-specific CD4\(^+\) T cells is associated with control of viraemia in HIV-infected individuals (Rosenberg et al., 1997).

However, the effector functions of FV-specific CD4\(^+\) T cells were lost 3 weeks after FV infection, with a dramatic drop in the frequency of IFN-\(\gamma\)-producing cells. Consequently, the donor cells were unable to reduce viral loads. Thus, although FV-specific CD4\(^+\) T cells expanded and upregulated the activation markers following adoptive transfer, the cells were functionally impaired at 3 weeks p.i. A similar downregulation in effector functions has already been shown for FV-specific CD8\(^+\) T cells with the effector phenotype during chronic FV infection (Dittmer et al., 2004). These cells fail to produce IFN-\(\gamma\) and the cytolytic molecules and hence are unable to degranulate and kill FV-labelled target cells in vivo (Zelinskyy et al., 2005). Contemporaneously with the development of CD8\(^+\) T-cell dysfunction, which already begins at 2–3 weeks p.i., is the peak expansion of Tregs (Zelinskyy et al., 2006). Several studies have also reasoned that the expansion of Tregs is a strategy to suppress anti-retroviral CTL responses (Iwashiro et al., 2001a; Dittmer et al., 2004, Estes et al., 2006).

In our study, the downregulation in IFN-\(\gamma\) production by FV-specific CD4\(^+\) T cells during acute FV infection was observed around the same time of peak Treg expansion. In order to elucidate the role of Tregs in inhibiting CD4\(^+\) T-cell responses, we used the DEREG mouse model. DEREG.Y10 mice infected with FV for 3 weeks had a sharp augmentation in IFN-\(\gamma\) production by adoptively transferred FV-specific CD4\(^+\) T cells after the depletion of Foxp3\(^+\) Tregs. Therefore, our results clearly provide substantial evidence for the immunosuppressive role of Tregs leading to CD4\(^+\) T-cell responses. This is in contrast with a recent finding, which emphasized that Tregs facilitate protective immunity to HSV infection (Lund et al., 2008). Lund et al. (2008) showed that ablation of Tregs in HSV-2-infected DEREG mice interfered with the trafficking of T cells to the vaginal mucosa, the site of viral replication. In contrast with HSV infection, the site of pathogen replication during FV infection is the lymphoid tissues. This might explain the differences in the outcome of the two studies. Nevertheless, our study clearly indicated that Tregs were involved in suppression of antiviral CD4\(^+\) T-cell responses during an acute retroviral infection. Additionally, we have recently shown that short-term depletion of Tregs did not result in severe immunopathology during acute FV infection (Zelinskyy et al., 2009).

From the current findings, it is conceivable that Th cells are indispensable for resistance to acute FV infection. Th cells mediate help to B cells and CD8\(^+\) T-cell responses during acute FV infection and therefore it becomes imperative to include Th cells in the development of retroviral vaccines. Moreover, our results also establish that Th cells have direct anti-retroviral effects on replicating virus and this anti-retroviral immunity can be suppressed by Tregs. Therefore, these aspects of CD4\(^+\) T-cell immune responses also need to be taken into account when designing anti-retroviral vaccines.

**METHODS**

**Mice.** Experiments were done using (H-2\(^b\)) (C57BL/10 × A.BY) F\(_1\) mice (Jackson Labs) that are resistant to FV-induced leukaemia. FV-susceptible (H-2\(^b\)) (C57BL/10.A × A.BY) F\(_1\) mice (Jackson Labs) were utilized for class-II tetramer studies. FV-specific CD4\(^+\) TCR\(\beta\)-tg mice, which carry a TCR transgene that is specific for the Env protein of F-MuLV (Antunes et al., 2008), were back-crossed with CD45.1-congenic C57BL/6 mice. DEREG mice (Lahl et al., 2007) (Tim Sparwasser, Rocky Mountain Labs, Montana, USA) were crossed once with (C57BL/10 × A.BY) F\(_1\) mice and were referred to as DEREG.Y10. The mice used were sex- and age-matched, and maintained under pathogen-free conditions.

**Virus and viral infection.** The FV stock was an FV complex containing B-tropic F-MuLV and polycythemia-inducing spleen focus-forming virus (Chesebro et al., 1990) (Bruce Chesebro, Rocky Mountain Labs, Montana, USA). The stock was prepared as a 15% spleen homogenate as described by Hasenkgrug et al. (1998b). The stock was free of contamination from LDV. Mice were infected intravenously with 0.5 ml PBS containing 6000–20 000 spleen focus-forming units of FV.

**In vivo T-cell depletion.** (C57BL/10 × A.BY) F\(_1\) mice were depleted of CD4\(^+\) T cells by injecting them intraperitoneally with 0.5 ml...
supernatant fluid obtained from hybridoma cell cultures for CD4-specific monoclonal antibody YTS 191.1. CD4 depletion was carried out at days −6, −4, −2 and at 0, 2 and 4 days p.i. with FV. Depletion of Tregs was carried out in DEREG.Y10 mice by injecting each mouse intraperitoneally on alternate days for 1 week with 1 μg DT (Merck) diluted in endotoxin-free PBS.

**IC assay.** The IC assay described by Robertson et al. (1991) was used to determine the number of IC in the spleen.

**Virus-neutralizing antibody assay.** The assay was performed as described by Morrison et al. (1987). The titre was defined as the plasma dilution at which >75% of F-MuLV was neutralized.

**F-MuLV-binding antibody ELISA.** ELISA plates (Nunc) were coated with whole F-MuLV antigen, blocked with fetal calf serum (Biochrom), and incubated with serum dilutions. Binding antibodies were detected using a polyclonal anti-mouse horseradish peroxidase-coupled anti-IgG antibody (Dianova) and the substrate 3,3′,5′-tetramethylbenzidine (Sigma-Aldrich). Sera were considered positive if the optical density at 492 nm was threefold higher than that obtained with sera from naive mice.

**Surface and intracellular staining, and flow cytometry.** Antibodies used for cell-surface staining were anti-CD4 (GK 1.5), anti-CD8 (1B10), anti-CD45.1 (A20), anti-CD69 (MCA367), anti-Vα2 (F7.2), anti-CD43 (1B11), anti-CD45.1 (A20), anti-CD69 (H1.293), anti-CD127 (SB199), anti-CD62L (MEL-14), anti-Mac-1 (WT.5), anti-Vβ2 TCR (B20.1) and Fc block anti-mouse CD16/CD32 (all from BD Biosciences). Dead cells were excluded by using 7-aminoactinomycin D (Molecular Probes). IFN-γ production (He et al., 2004; Peterson et al., 2000) and Foxp3 expression were detected by intracellular staining using the Foxp3 staining kit (eBioscience) as described by Zelinsky et al. (2006). Data were acquired on a FACSScAlibur and a LSR II flow cytometer (Becton Dickinson). Analyses were done using Becton Dickinson CellQuest Pro and FlowJo software version 7.2.5 (Tree Star).

**Adoptive cell transfers.** The midi MACS Separation system (Miltenyi Biotec) was used for the isolation of CD4+ T cells. The purity of the cell fractions was >90% in all cases. The enriched fraction of CD4+ T cells was stained for CD43 and CD62L to verify their naive phenotype. Adoptive transfers were done by intravenous injection of 7.5 × 10^6 cells in PBS. Recipient mice were infected with FV and received CD4+ T cells at 3, 14 or 21 days p.i. Seven days post-transfer, spleen samples were assayed for viral load and expression of IFN-γ, Foxp3 and surface markers.

**Tetramers and tetramer staining.** MHC class-II tetramers loaded with I-Ab-restricted FV-specific CD4+ T-cell epitope (H19-Env; EPITLSLTPRNCWNLKL) were obtained from T. Schumacher (National Cancer Institute, Amsterdam) and the NIH Tetrramer Facility (Atlanta). Tetramers were used for detection of I-Ab-restricted FV Env-specific CD4+ T cells. Nucleated spleen cells were incubated with PE-labelled I-Ab tetramers for 2–3 h at 37 °C and later stained with anti-CD4 and anti-Mac-1 for 15 min at room temperature. For the detection of activated virus-specific CD8+ T cells, spleen cells were stained with anti-CD8, anti-CD43 and PE-labelled MHC class I H-2Dd tetramers specific for the immunodominant GagL CTL epitope gPr80885–93 (Zelinsky et al., 2006).

**Statistical analyses.** Statistical analyses and graphical presentations were computed with GraphPad Prism version 5. Statistical differences (P values) were analysed by using the Mann–Whitney U test.

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