TGF- β Blockade Does Not Improve Control of an Established Persistent Viral Infection

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Abstract

Acute resolving viral infections are often associated with a strong and multi-specific T-cell response, whereas in persistent viral infections T-cell responses are often impaired. It has been suggested that the resuscitation of the antiviral T-cell response could be a powerful tool to target persisting viruses. Several immunoregulatory pathways, such as IL-10 and TGF- β , have been shown to be involved in the induction of T-cell exhaustion and viral persistence. In this study, we sought to investigate whether TGF- β signaling is also relevant in the maintenance of T-cell exhaustion after viral persistence has been established, and whether blockade of TGF- β signaling could improve control of viral replication in a mouse model of persistent virus infection. Using the LCMV clone 13 model, we analyzed the frequency, function, and phenotype of virus-specific CD4 and CD8 T cells following therapeutic TGF- β signaling blockade. We show that *in vivo* blockade of the TGF- β receptor failed to substantially enhance the antiviral T-cell response, and was insufficient to mediate a therapeutically-relevant reduction of viral titers in different tissues. Thus, although TGF- β signaling has the ability to hamper antiviral immunity, its pharmacological blockade may not be sufficient to tackle persistent viruses.

Introduction

PERSISTENT INFECTIONS WITH VIRAL PATHOGENS SUCH AS HEPATITIS VIRUSES B AND C (HBV AND HCV), or the human immunodeficiency virus (HIV), pose major threats to global health. Currently, the available treatment options are rather limited and can cause serious side effects (1,2). Immunotherapeutic interventions present an interesting approach to tackle viruses that have successfully established persistence. In order to design such interventions, much effort has been made to understand why certain viruses are able to circumvent the host's immune response. It was found that several immunoregulatory or inhibitory pathways are involved in hampering the virus-specific immune response (i.e., mice with absent or reduced signaling of interleukin-10 [IL-10], or transforming growth factor- β [TGF- β], effectively cleared an otherwise persistent virus from all tissues) (3–5). In both cases, viral clearance was associated with strong and multi-specific antiviral T-cell responses, and the development of functional T-cell memory that protected from a secondary challenge (3,5). It was shown that IL-10 mediates its immunosuppressive effects predominantly through inhibition of CD4 T cells (6), whereas TGF- β signaling induces dysfunction and

apoptosis in the virus-specific CD8 T-cell compartment (3). In order to evaluate the therapeutic implications of those findings in an established persistent infection, several studies have focused on blocking the IL-10 signaling pathway alone or in combination approaches (7,8). However, the therapeutic potential of TGF- β blockade after establishment of viral persistence has thus far not been evaluated.

The majority of the described studies have been performed using a well-defined mouse model of persistent virus infection, the lymphocytic choriomeningitis virus (LCMV) clone 13 model. This is an interesting model, since it mirrors several aspects of chronic virus infections in humans (9). Indeed, signs of T-cell exhaustion, such as expression of defined inhibitory molecules on effector T cells and impaired secretion of antiviral cytokines, can be found in both murine and human chronic viral infection (10–14). In this study, using the LCMV clone 13 model, we analyzed the effects of TGF- β receptor blockade on the antiviral T-cell response and on viral titers after establishment of viral persistence. We observed that transient therapeutic TGF- β signaling blockade failed to markedly impact the virus-specific T-cell response regarding their frequency, phenotype, and function. Moreover, TGF- β blockade did not result in improved control of viral replication.

Materials and Methods

Mice and virus

C57BL/6 mice obtained from The Jackson Laboratory, and LCMV-GP33 TCR transgenic P14 mice, were used in this study. All mice were housed at the La Jolla Institute for Allergy and Immunology (LIAI) under specific pathogen-free conditions. All experiments performed for this study were approved by the LIAI Animal Care committee (protocol no. AP100-MvH and AP152-MvH). LCMV variant clone 13 was used in all experiments, and stocks of the virus were prepared as previously described (4). Mice 6–12 wk of age were infected with a single dose of 2×10^6 plaque-forming units (PFU) intravenously.

TGF- β blockade

The small molecule SB 431542 has previously been shown to act as a TGF- β receptor I blocker that effectively reduces smad3 phosphorylation (15), and has successfully been used to block TGF- β signaling *in vivo* (16–18). SB 431542 (Tocris Bioscience, Ellisville, MI) was dissolved in 100% DMSO and administered intraperitoneally to persistently infected mice starting from day 21 post-infection at a dose of 400 μ g (50 μ L of a 19 μ mol solution) every 2–3 d for a total of 4 to 5 treatments. In one control experiment, mice additionally received 4–5 doses of 250 μ g of a TGF- β antibody intraperitoneally (clone 1D11.16.8; BioXcell, West Lebanon, NH), which has been widely used to block TGF- β *in vivo* (19–22). As we have not observed any differences between SB 431542-treated and SB 431542 + 1D11.16.8-treated mice in the parameters analyzed in this study, we have integrated the results from the control experiments into the graphs throughout the article. Control animals received 50 μ L DMSO, herein referred to as placebo, and 250 μ g of rat IgG₁ isotype antibody in the combination experiment (BioXCell).

Flow cytometry

Splenocytes were harvested at the indicated time points and single-cell suspensions were prepared. The cells were stained with fluorescence-labeled surface antibodies against CD4, CD8a, CD19, PD-1, LAG-3, 2B4, KLRG-1, CD127, CD45.1, and CD45.2 (BD Biosciences, Franklin Lakes, NJ; eBioscience, San Diego, CA; BioLegend, San Diego, CA) as indicated. For selected experiments, cells were stained with H-2DbGP33 and H-2DbNP396 class I pentamers (Proimmune Inc., Sarasota, FL), according to the manufacturer's instructions prior to surface staining. All cells were fixed in 2% cold PFA prior to acquisition on an LSRII flow cytometer. For intracellular stains, splenocyte single-cell suspensions were stimulated for 5 h with 5 μ g/mL MHC class I-restricted viral peptides (GP33 or NP396), or 10 μ g/mL MHC class II-restricted viral peptide (GP61) in the presence of brefeldin A (Sigma-Aldrich, St. Louis, MO), and anti-CD107a antibody (for selected experiments). Following stimulation, the cells were stained for surface markers, fixed, and permeabilized with Cytotfix/Cytoperm (BD Biosciences), and stained for intracellular interferon- γ (IFN- γ), IL-2, and tumor necrosis factor (TNF). Cells were acquired on an LSRII flow cytometer and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

TGF- β ELISA

TGF- β enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN) was performed according to the manufacturer's instructions, and analysis was performed on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA).

Analysis of viral titers

Organs were snap-frozen on dry ice, weighed, and homogenized. For plaque assay, five different dilutions of each homogenized organ or serum sample were prepared and plated on six-well plates coated with Vero cells in duplicate. After 5–6 d, the wells were fixed with 3.7% formaldehyde and stained with crystal violet. Plaques were counted and PFU numbers were calculated for at least two different dilutions. Quantitative real-time polymerase chain reaction (PCR) was performed as previously described (23). Briefly, RNA was isolated from serum or homogenized tissue samples using RNAqueous (Ambion, Foster City, CA). Next, cDNA synthesis from purified RNA was performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and a gene-specific primer (GP-R). An SYBR Green kit (Hoffmann-La Roche AG, Basel, Switzerland) was used for quantitative real-time PCR reaction on a Light Cycler 480 (Hoffmann-La Roche AG). The pSG5-GP plasmid (24) used to create a standard curve was a gift from J.C. de la Torre (Scripps Research Institute, San Diego, CA). Viral LCMV stock was used as a positive control in both assays.

Statistical analysis

All error bars in dot plots and bar graphs represent the standard deviation. Whisker plots are shown as minimum to maximum *p* values, and were calculated using the two-tailed Student's *t*-test with Prism software (GraphPad Software, La Jolla, CA). We have performed a total of four independent experiments with 3–5 mice per group in each experiment. All parameters were studied in at least two separate experiments, with the exceptions of the data using TCR transgenic cells (one experiment with 4 mice per group), and the TGF- β ELISA (one experiment with 3–5 mice per time point; samples were run in duplicate).

Results

Effects of TGF- β blockade on antiviral CD8 T cells

To address the question of whether TGF- β signaling is important to maintain functional T-cell exhaustion during an already established chronic virus infection, we infected C57BL/6 mice with LCMV clone 13. When challenged with 2×10^6 PFU of this LCMV isolate, mice develop prolonged viremia in various organs (25). The CD8 T-cell responses to LCMV-clone13 in H-2D^b mice are characterized by the deletion of T cells that recognize the high-affinity epitope NP396 (26), which constitutes the immunodominant CD8-restricted epitope during acute LCMV-Armstrong infection (27). Moreover, LCMV clone 13 infection results in functional exhaustion of other immunodominant and subdominant epitopes, including GP33 and GP276 (28). Previously it was shown that mice expressing a dominant negative form of the TGF- β receptor II (dnTGFBR^{II} mice) did not display this

T-cell phenotype usually observed during clone 13 infection (3). Indeed, these mice were capable of clearing the virus from all tissues, and did not show phenotypical signs of T-cell exhaustion. Those findings established a strong role for TGF- β in the induction of T-cell dysfunction during chronic LCMV infection. In order to address whether TGF- β could also have a role in maintaining T-cell dysfunction, we analyzed plasma levels of TGF- β over time in mice infected with LCMV clone 13, and found increasing TGF- β levels over the first 3 wk post-infection (Fig. 1B). Next, we performed blocking experiments *in vivo* to determine whether TGF- β has functional relevance in maintaining virus-specific T-cell exhaustion during persistent infection. In order to be readily

translatable to humans, therapeutic interventions in persistent infections have to be initiated after establishment of persistence, as chronic virus infections are typically diagnosed at this stage (2). We decided to initiate the treatment 21 d post-infection, as T-cell exhaustion is fully established by then and viral titers are still high (8,11). TGF- β signaling blockade was performed by treating persistently-infected mice with SB 431542, a small molecule previously shown to potentially block the TGF- β receptor type 1 (15). Four to 5 doses were administered over a period of 10 d; 7 or 14 d after the last treatment, T-cell responses and viral titers were analyzed (Fig. 1A). When analyzing the virus-specific CD8 T-cell responses by pentamer staining, we found higher percentages

FIG. 1. Plasma TGF- β levels increase over time during LCMV clone 13 (cl13) infection, and TGF- β R blockade enhances the frequency of virus-specific CD8 T cells in the spleen. (A) Diagram of experimental design. For selected experiments, C57BL/6 recipient mice were seeded with LCMV-TCR transgenic cells. Starting on day 21 post-LCMV clone 13 infection, mice received 4–5 treatments of either TGF- β R blockade or placebo every 2–3 d. For most experiments, serum and organs were harvested for analysis 7 d after the last treatment. Experiments performed 14 d after completion of the treatment are specifically labeled. (B) Plasma-levels of TGF- β 1 in naïve mice (black box), and LCMV clone 13-infected untreated mice (grey boxes), were measured by ELISA on the indicated days post-infection. (C) Pentamer stainings revealed higher frequencies of GP33-specific CD8 T cells after TGF- β blockade. Two representative pseudocolor plots gated on CD8⁺CD19[−] T cells are shown. (D) Frequencies of NP396-specific CD8 T cells were not affected by TGF- β R blockade. (E) Overall CD8 T-cell numbers were not affected by TGF- β R blockade (**p* < 0.05).

of GP33-specific CD8 T cells in anti-TGF- β receptor (α -TGF- β R)-treated mice compared to placebo-treated mice (Fig. 1C). Not surprisingly, TGF- β R blockade did not affect the frequency of NP396-specific cells (Fig. 1D), as this population is largely deleted early after LCMV clone 13 infection. Total numbers of CD8 T cells per spleen were nearly identical in both groups (Fig. 1E, $p=0.99$).

Effects on virus-specific cytokine production and degranulation

Next, we sought to analyze whether TGF- β receptor blockade would also increase the frequency of cytokine-producing cells, and whether it enables virus-specific cells to produce multiple cytokines at the same time. The loss of the ability to produce multiple cytokines has been associated with CD8 T-cell exhaustion during chronic virus infection (29). We

analyzed the production of IFN- γ , TNF, and IL-2 following *in vitro* stimulation with two CD8-restricted (GP33 and GP276), and one CD4-restricted (GP61) epitope. Although we found that TGF- β R blockade resulted in higher numbers of GP33-specific IFN- γ -producing CD8 T cells, we did not detect significant differences in TNF or IL-2 production from those cells (Fig. 2A and B). However, this was not associated with alterations in the cytokine secretion pattern of virus-specific T cells, as we found similar percentages of single-cytokine and multiple-cytokine producers in both groups (Fig. 2C). Also, it did not affect the virus-specific degranulation capacity of the CD8 T cells during *in vitro* stimulation as analyzed by CD107a expression (Fig. 2D). When analyzing the virus-specific cytokine production by GP276-specific CD8 T cells and GP61-specific CD4 T cells, we did not observe a statistically relevant difference in treated animals compared to control mice (Fig. 2E and F). Of note, the overall numbers of both CD4 and CD8

FIG. 2. Virus-specific secretion of IL-2, IFN- γ , and TNF following TGF- β R blockade. (A) Shown is the frequency of CD8⁺ cells that produce IL-2, IFN- γ , or TNF following stimulation with the GP33 peptide. (B) Representative pseudocolor plots from intracellular cytokine stainings gated on CD8 T cells. (C) Pie charts display the cytokine secretion pattern of the cells displayed in A. (D) Frequency of CD8 T cells expressing the degranulation marker CD107a during 5 h incubation with the GP33 peptide. (E) Frequency of CD8⁺ cells that produce IL-2, IFN- γ , or TNF, following stimulation with the GP276-peptide. (F) Frequency of IL-2-, IFN- γ -, or TNF-producing CD4 T cells following stimulation with the GP61 peptide (* $p < 0.05$). Color images available online at www.liebertonline.com/vim

T cells in spleens from treated mice did not differ from T-cell numbers in control mice.

Adoptive transfer studies of TCR transgenic CD8 T cells

To analyze the virus-specific CD8 T-cell compartment more closely following LCMV clone 13 infection and therapeutic TGF- β receptor blockade, we transferred small numbers of naïve CD45.1 TCR transgenic CD8 T cells (P14s) into CD45.2 hosts prior to viral infection. Using this approach, a distinct virus-specific CD8 T cell population can be specifically tracked and its functional and phenotypical determinants can be analyzed. Following TGF- β -receptor blockade, we recovered significantly higher numbers of transgenic P14 cells in the spleens of persistently infected mice compared to

placebo treatment (Fig. 3A). However, the percentage of IFN- γ -producing cells within the P14 cells was only slightly increased in the anti-TGF- β receptor group (Fig. 3B). Next, we wanted to assess whether the transferred P14 cells displayed changes in the expression pattern of defined surface markers in the Vero- versus placebo-treated mice. The expression of the interleukin-7 receptor alpha chain (CD127) has been associated with long-lived, memory-like T cells, whereas the expression of the killer cell lectin-like receptor subfamily G member 1 (KLRG1) has been linked to short-lived, terminally-differentiated effector cells (30,31). As shown in Fig. 3C, TGF- β receptor blockade did not alter the expression of CD127 or KLRG1 on P14 cells, suggesting that the higher number of P14 was not restricted to a certain subpopulation within the virus-specific CD8 T cells. Moreover, we did not observe differences in the expression of the inhibitory

FIG. 3. Enhancement of CD8 T-cell responses through TGF- β signaling blockade is insufficient to improve control of viral replication. (A) About 2000 naïve GP33–41 transgenic P14 cells were injected into B6 mice 1 d prior to LCMV clone 13 infection. Significantly higher numbers of P14 cells could be recovered in the spleens of treated mice. (B) Percentage of IFN- γ -producing cells among GP33-specific P14 cells. (C) Expression of defined surface molecules on P14 cells. (D) Viral titers were analyzed before and 14 d after completion of the TGF- β blockade in the serum of infected mice. (E) At 7 d after completion of the therapeutic TGF- β blockade, viral titers were analyzed in serum, liver, and kidney, by plaque assay or qPCR (* p < 0.05).

receptors programmed death 1 (PD-1), or the NK cell receptor 2B4 (or CD244) (Fig. 3C), surface markers that are commonly used to describe exhausted T cells (29).

TGF- β R blockade does not improve immune-mediated control of viral replication

Previous reports have shown that clone 13 persists in various organs, and while in serum and some organs such as liver and spleen that titers decrease over months; however, high titers remain detectable in other organs, particularly the kidney. Recently it has been shown that it is indeed possible to accelerate the decline of viral titers in the serum and other organs by enhancing CD8 T-cell immunity (11,32). In order to determine whether the enhanced CD8 response to the virus that we observed following TGF- β R blockade is also capable of accelerating the reduction of viral titers in the serum, we obtained serum samples from infected mice before initiation and 2 wk after termination of the treatment, and analyzed viral titers. However, in both groups, viral titers decreased to the same extent (Fig. 3D). We made similar observations when we analyzed serum, liver, and kidney samples 1 wk after termination of the treatment (Fig. 3E).

Discussion

The importance of the T-cell response in the clearance of various pathogens is clearly established. Thus in recent years various reports have focused on the resuscitation of virus-specific immune responses in order to achieve clearance of a persistent viral pathogen (7,8,11, 32–34). Blockade of various immunosuppressive pathways such as IL-10, PD-1, or Tim-3, or direct boosting of the cellular immune response through cytokine treatment or vaccinations, have been performed alone or in different combination approaches (7,8,11,32,33). Indeed, some of these interventions were found to be powerful enough to augment the virus-specific T-cell response, even after the virus had already established persistence. In some cases, this resulted in an acceleration of virus control in treated mice (7,8,11,32,33), suggesting that T-cell-based immunotherapies might be an interesting approach to tackle chronic viral infections in humans.

The observations made by Tinoco *et al.* (3), that TGF- β signaling in virus-specific T cells enables T-cell exhaustion and persistent infection, raised the question of whether TGF- β could be targeted therapeutically in chronic viral infections. In order to analyze the therapeutic implications of these important findings, we performed TGF- β signaling blockade experiments in a murine model of persistent viral infection. In order to be translatable to chronic viral infections in humans, we initiated therapeutic TGF- β signaling blockade after T-cell exhaustion and virus persistence had been established. Following TGF- β R signaling blockade, we observed a modest increase in the number of GP33-specific CD8 T cells that could be recovered from the spleens of treated mice compared to control mice. Although this finding is in agreement with the findings by Tinoco *et al.*, that TGF- β signaling during LCMV clone 13 infection induces apoptosis of effector T cells (3), we did not observe those differences for other CD8 T-cell epitopes. Moreover, it was shown that dnTGF β RII mouse-derived CD8 T cells displayed slightly stronger IFN- γ production upon *in vitro* stimulation compared to WT CD8 T cells [5.8% versus 4% for GP33 (3)],

whereas they did not observe relevant differences in PD-1 expression on these cells. We made similar observations using adoptive transfer experiments with TCR transgenic CD8 T cells (P14). These experiments revealed that TGF- β signaling blockade marginally affected the percentage of cytokine-producing cells within the virus-specific CD8 T-cell population (Fig. 3B), and that cells from treated mice were phenotypically indistinguishable from their untreated counterparts regarding the expression of CD127, KLRG1, PD-1, and 2B4 (Fig. 3C).

As TGF- β blockade failed to substantially improve the antiviral T-cell response, it is not surprising that viral titers remained unaffected by this intervention. Importantly, this was true for viral titers in solid organs as well as in the serum, which argues against site-specific effects of TGF- β blockade.

Collectively, our data suggest that TGF- β might contribute to the maintenance of T-cell dysfunction during persistent viral infection. However, blockade of TGF- β signaling after the establishment of virus persistence does not improve the host's ability to control viral replication.

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Author Disclosure Statement

No competing financial interests exist.

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