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ORIGINAL ARTICLE

Chronic retroviral infection of mice promotes tumor development, but CD137 agonist therapy restores effective tumor immune surveillance

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Abstract

T cell responses are crucial for anti-tumor immunity. In chronic viral infections, anti-tumor T cell responses can be compromised due to various immunological mechanisms, including T cell exhaustion. To study mechanisms of anti-tumor immunity during a chronic viral infection, we made use of the well-established Friend virus (FV) mouse model. Chronically FV-infected mice are impaired in their ability to reject FBL-3 cells—a virus-induced tumor cell line of C57BL/6 origin. Here we aimed to explore therapeutic strategies to overcome the influence of T cell exhaustion during chronic viral infection, and reactivate effector $CD8^+$ and $CD4^+$ T cells to eliminate tumor cells. For T cell stimulation, agonistic antibodies against the tumor necrosis factor receptor (TNFR) superfamily members CD137 and CD134 were used, because they were reported to augment the cytotoxic program of T cells. αCD137 agonistic therapy, but not αCD134 agonistic therapy, resulted in FBL-3 tumor elimination in chronically FV-infected mice. CD137 stimulation significantly enhanced the cytotoxic activity of both $CD4^+$ and $CD8^+$ T cells, which were both required for efficient tumor control. Our study suggests that agonistic antibodies to CD137 can efficiently enhance anti-tumor immunity even in the setting of chronic viral infection, which might have promising therapeutic applications.

Keywords Costimulatory molecule · Anti-tumor immunity · Agonistic antibody · Friend retrovirus · Effector T cells

Abbreviations

EL-4	Chemically induced tumor cell line
Eomes	Eomesodermin
FBL-3	Friend virus-induced tumor cell line
F-MuLV	Friend murine leukemia virus
FV	Friend virus
FVD	Fixable viability dye
GzmB	Granzyme B
Tet	Tetramer

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Introduction

T cell responses are crucial for anti-tumor immunity. They can effectively eliminate benign or malignant tumor cells before they cause disease. Evidently, the most important role in destroying transformed cells belongs to cytotoxic $CD8^+$ T cells, although $CD4^+$ T cells are not only offering help to $CD8^+$ T cells and antibody responses, but can also mediate direct anti-tumor effector functions. For example, in melanoma mouse models and the Friend virus leukemia model tumor control has been attributed to direct MHC class II-dependent $CD4^+$ T cell killing of tumor cells [1–3].

In chronic virus infections, anti-tumor T cell responses can be compromised due to various immunological events, such as reduced numbers of T cells or T cell exhaustion. Regulatory T cells (Tregs) become activated and expand during the course of many chronic infections, which is one

reason why effector T cells lose their ability to control tumor growth [4]. It was also previously reported that Tregs themselves contribute to tumor progression as they permeate into tumor tissue and draining lymph nodes [5].

Impairment of anti-tumor immunity during the chronic phase of infections has been described in many viral infections including HIV [6, 7]. Not only the incidence of AIDS-defining cancers, such as Kaposi sarcoma, primary central nervous system lymphoma, non-Hodgkin lymphoma, and cervical cancer, have high prevalence even in HIV-infected patients receiving antiretroviral therapy compared to the general population; but also morbidity and mortality linked to non-AIDS-defining cancers, e.g. melanoma, are significantly higher in HIV-positive individuals [8].

To study the mechanisms of immunity during a chronic retroviral infection, we made use of the well-established Friend virus (FV) mouse model. FV causes lethal erythroleukemia in susceptible mouse strains; however, resistant strains, such as C57BL/6, do not develop the disease in part due to their rapid immune response against the virus. Nevertheless, these mice cannot clear the virus completely and develop a chronic retroviral infection. Such mice can be used to study the mechanisms of immunity during the chronic phase of a virus infection. If these chronically FV-infected mice are challenged with tumor cells, they are impaired in their ability to reject neoplasm [9]. As a tumor model, we have used the FBL-3 tumor (a FV-transformed tumor cell line of C57BL/6 mouse origin) that can be implanted subcutaneously (s.c.) and grows locally. In non-infected C57BL/6 mice, the tumor grows for several days before it is completely rejected by T cells [3]. This evidence comes from experiments in which $CD8^+$ T cells were depleted in FBL-3 challenged mice resulting in tumor growth [10]. Moreover, even though $CD4^+$ T cells are not as important as $CD8^+$ T cells for FBL-3 tumor rejection, co-stimulation of $CD4^+$ T cells in $CD8^+$ T cell-depleted mice restored FBL-3 tumor control [10]. In contrast, in chronically FV-infected mice, tumor rejection is severely impaired and mice have to be killed due to uncontrolled tumor growth. It has previously been shown that during chronic FV infection activated Tregs interfere with anti-tumor effector T cell responses [11]. Hence, we aimed to explore therapeutic strategies to overcome the influence of Tregs and other T cell exhaustion mechanisms during chronic viral infection and reactivate the ability of effector $CD8^+$ and $CD4^+$ T cells to eliminate tumor cells and prevent tumor growth.

To address the question if the anti-tumor capacity of T cells can be restored during chronic viral infection, we made use of agonistic antibodies against the tumor necrosis factor receptor (TNFR) superfamily members CD137 and CD134, which were described as promising targets for enhancing anti-tumor immune response [12–14]. The first study on CD137 anti-tumor therapy revealed the eradication of large tumors in mice through the augmented cytotoxicity of T cells [15]. Treatment with agonistic antibodies targeting CD137 has been shown to be involved in the functional improvement of $CD8^+$ and $CD4^+$ T cells, including their reinvigoration from functional exhaustion [16] as well as in the activation of natural killer and dendritic cells [17]. Moreover, it was noted that CD137 in combination with other anti-cancer drugs strongly promotes the establishment of tumor-specific memory T cells [18].

Administration of CD134 antibody has been described to contribute to tumor antigen-specific cytotoxicity of $CD8^+$ T cells as well as to the expansion and effector function of memory $CD4^+$ T cells [19, 20]. However, it remains elusive if CD137 and CD134 agonistic treatment can contribute to T cell reactivation during a chronic viral infection and whether this may influence anti-tumor immunity in this specific environment.

In the present study, we demonstrate that the treatment of chronically FV-infected mice with αCD137, but not αCD134 agonistic antibody, resulted in effective anti-tumor T cell responses, control of tumor growth, and complete tumor regression. Moreover, we defined the cell populations responsible for that regression. Our results reveal important evidence of effective tumor treatment with agonistic antibody therapy during chronic viral infection.

Materials and methods

Virus and viral infection

The FV stock used in the experiments was a FV complex containing B-tropic Friend murine leukemia helper virus (F-MuLV) and polycythemia-inducing spleen focus-forming virus. The stock was prepared as described in detail in [21].

Tumor challenge

A total of 1×10^7 FBL-3 tumor cells were injected s.c. on the right flank of mice in 100 µl PBS on day 42 post FV infection. Tumor size was calculated by the formula that had been previously described in [10].

In vivo cell depletion and CD134/CD137 agonistic treatment

The anti-CD134 (clone OX-86) and anti-CD137 (clone LOB 12.3) antibodies were purchased from BioXCell. Each antibody was administered i.p. every second day starting at day 43 post infection in the dose of 100 µg. NK cells were depleted by i.p. injection of 150 µl of supernatant fluid containing NK1.1-specific mAb PK136. The depletion antibody was injected 1 day prior to in vivo cytotoxicity assay.

The depletion of $CD4^+$ or $CD8^+$ T cells started one day prior to tumor inoculation and was carried out every second day until mice were killed because of the progressive tumor growth. The $CD4^+$ and $CD8^+$ T cells were depleted by injections of 100 µl supernatant fluid obtained from hybridoma cell culture YTS 191.1 or 169.4 producing CD4-specific mAb or CD8a-specific mAb, respectively. The depletion ablated more than 95% of $CD4^+$ and $CD8^+$ T cells, whereas $CD8^+CD11c^+$ dendritic cells were reduced by only 15–50% in different organs [22].

Flow cytometry

Cell surface and intracellular stainings were performed using antibodies as follows: CD4 (RM 4–5; BioLegend), CD8 (53-6.7; eBioscience), CD43 (1B11; BioLegend), CD62L (MEL-14; BioLegend), Foxp3 (NRRF-30; eBioscience), granzyme B (GB11; BioLegend) and Eomesodermin (Dan-11mag; eBioscience). Dead cells were excluded by using Fixable Viability Dye (FVD) (eBioscience). BD Cytofix/Cytoperm Fixation/Permeabilization kit or Foxp3 staining kit (eBioscience) was used for intracellular staining following the manufacturer's instructions. To determine intracellular production of IFNγ, cells were restimulated with ionomycin (500 ng/ml), phorbol myristate acetate (PMA; 25 ng/ml), monensin (1X), and brefeldin A (2 µg/ml) diluted in IMDM buffer and incubated for 3 h at 37 °C. Cells were fixed and permeabilized. After that, cells were labeled with mAbs specific for IFNγ (APC, XMG1.2, eBioscience). The tetramers specificity and tetramer staining have been previously described in detail in [21, 22]. Data were acquired at LSR II flow cytometer (BD Biosciences). The analyses were done using FlowJo 7.6 software (Tree Star Inc., Ashland, OR).

Infectious center assay

Single cell suspensions from infected mouse lymph nodes and spleens were plated onto susceptible *Mus dunni* cells and incubated at 37 °C and 5% CO_2 for 3 days. Cells were then fixed with 95% ethanol, stained with F-MuLV envelope-specific mAb 720, and developed with a peroxidase-conjugated goat anti-mouse antibody and aminoethylcarbazol for the detection of foci.

In vivo cytotoxicity assay

The tumor target killing assay was performed using 5×10^5 tdTomato FBL-3 cells/mouse and 5×10^5 EL-4 cells/mouse labeled with 10 µM Violet cell tracer (BioLegend). FBL-3 and EL-4 cells, mixed in ratio 1:1, were injected i.p. into the mice of interest. In addition, one day prior to injection of target cells, all mice received an NK cell depletion antibody. The mice were killed 4 h after cell transfer, and i.p. lavage of the peritoneal cavity was performed with 10 ml PBS to obtain the cells. The cells were washed, resuspended in buffer, containing FVD, and analyzed by flow cytometry. The ratio of $tdTomato^+$ FBL-3 target cells versus $Violet^+$ EL-4 cells was calculated and normalized to the ratio in the naïve animals.

Statistical analysis

Statistical analysis and graphical presentations were performed with GraphPad Prism version 5. Statistical differences (p value) between the two groups were calculated using an unpaired t test. In this work, all p values ≤ 0.05 were determined to be significant.

Results

CD137, but not CD134 agonistic therapy in FV chronically infected mice mediates FBL-3 tumor cell rejection

Prior studies have shown that anti-tumor immunity in naïve C57BL/6 mice is efficient to completely eliminate FBL-3 tumor cells [3], and this protection is mediated by T cells. To verify that the anti-tumor activity of T cells is lost during chronic viral infection due to exhaustion mechanisms, we injected FBL-3 cells into chronically FV-infected mice and measured the tumor size over 15 days (Fig. 1a). Our results confirmed that the anti-tumor immunity is impaired during the chronic phase of FV infection and that FBL-3 tumor growth is not efficiently controlled. In order to determine if anti-tumor immune responses can be restored by costimulatory therapy, chronically FV-infected tumor-bearing mice were administered CD137 or CD134 agonistic antibodies every second day starting at day 1 after tumor inoculation. The tumor growth in mice that received CD134 agonistic treatment varied between animals, but overall was not different from the untreated control group (Fig. 1b). In contrast, the treatment with CD137 agonistic antibody resulted in tumor rejection in almost all mice (Fig. 1c). At day 15 post tumor inoculation, the tumor size in the αCD137-treated mice was significantly lower compared to untreated controls, whereas no significant difference was found after αCD134 treatment (Fig. 1d). This experiment suggested that anti-tumor immunity can be restored by CD137 agonistic antibody therapy in chronically infected mice.

Numbers of FV-specific $CD8^+$ T cells increase following αCD137 therapy

Important immunodominant T cell epitopes are shared between FV and FBL-3 tumor cells [23, 24]. Thus, we quantified FV-specific $CD4^+$ and $CD8^+$ T cell responses using

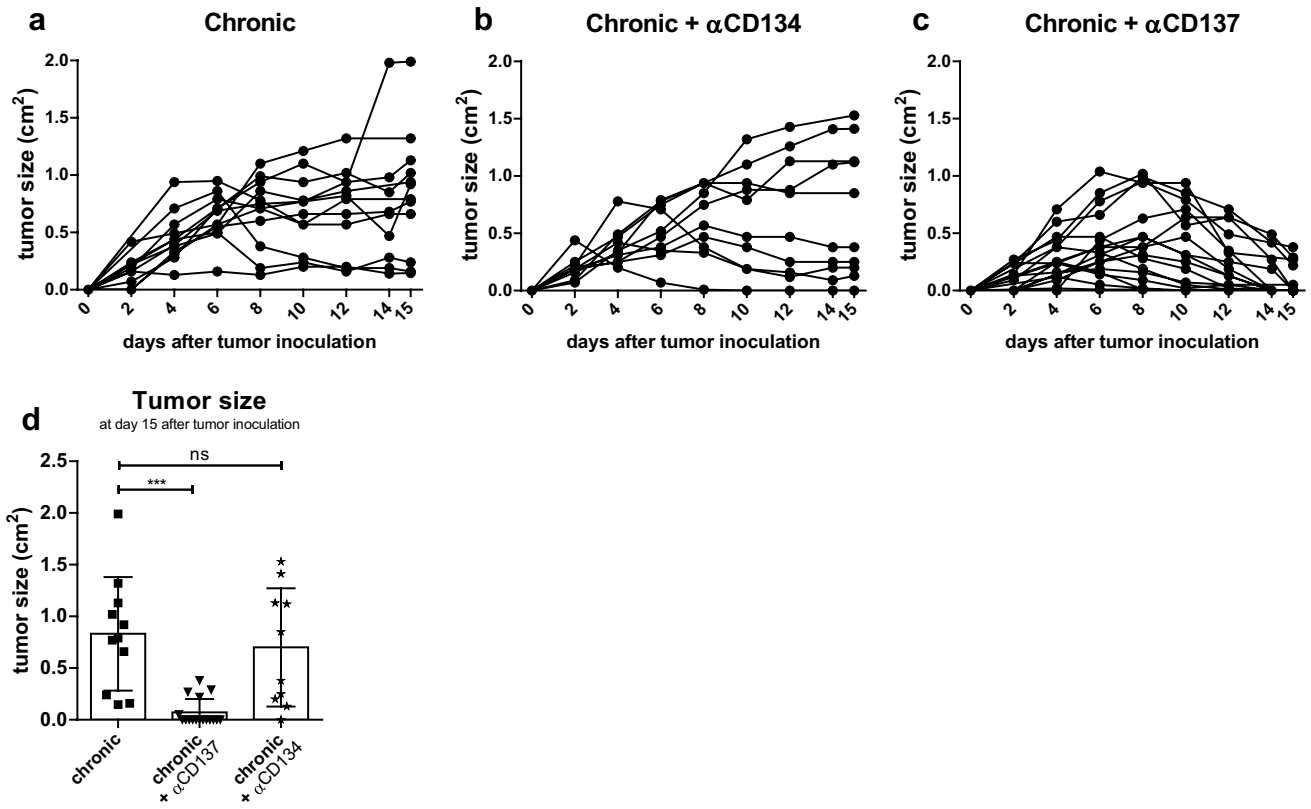


Fig. 1 Effect of αCD137 and αCD134 therapy on tumor formation during the chronic phase of FV infection. C57BL/6 mice chronically infected with FV were injected s.c. with 1×10^7 FBL-3 cells. Tumor size was measured every day. **a** FBL-3 cells injected into chronically FV-infected mice without treatment. Each line represents tumor progression in an individual mouse. **b** FBL-3 cells injected into chronically FV-infected mice receiving αCD134 treatment. **c** FBL-3 cells injected into chronically FV-infected mice receiving αCD137 treatment. **d** Tumor size in every group at day 15 after tumor inoculation. Each dot represents an individual mouse. Statistically significant differences between the groups were determined by the unpaired *t* test: $*p<0.05$; $**p<0.005$; $***p<0.0001$; ns, not significant. Data were pooled from three independent experiments

MHC class II and class I tetramers (Tet), respectively. In previous studies, it had been shown that FV-specific T cells remain detectable during the chronic phase of infection [21, 22].

Indeed, low absolute numbers of FV-specific $CD4^+$ and $CD8^+$ T cells were detected in most of the analyzed organs (spleen, non-draining lymph nodes, and draining lymph node) of chronically infected mice at day 15 post tumor challenge. Interestingly, CD137 agonist therapy significantly enhanced absolute numbers of Tet I^+CD8^+ T cells, whereas absolute numbers of Tet II^+CD4^+ T cells were not affected by the therapy (Fig. 2).

αCD137 therapy results in expansion of $CD8^+$ and $CD4^+$ T cells with a cytotoxic phenotype

As a next step, immunological correlates of protection for the agonistic CD137 therapy were determined. Since FBL-3 cells are known to be predominantly controlled by cytotoxic T cells and T helper 1-type cytokines, we analyzed markers for cytotoxicity and IFNγ production by $CD4^+$ and $CD8^+$ T cells. In mice, the cytotoxic program of T cells depends on the T-box transcription factor Eomesodermin (Eomes) that controls the production of granzymes and perforin by effector cells [25]. However, Eomes expression is reduced in chronically FV-infected mice [4]. Moreover, $CD4^+$ T cells are mostly non-cytotoxic and usually do not express Eomes [26]. During chronic FV infection, cytotoxic $CD4^+$ T cells can be found, but they kill infected target cells via the Fas/Fas ligand pathway [21]. It was, therefore, not surprising that only very few $CD4^+$ T cells in chronically FV-infected mice expressed Eomes (with no difference to the background levels of naïve control mice that were not infected nor tumor challenged), and the percentages of $Eomes^+$ $CD8^+$ T cells were also very low in the three analyzed organs—spleen, lymph nodes, and tumor-draining lymph node (Fig. 3a). In contrast, after agonistic CD137 therapy significantly more Eomes-expressing activated ($CD43^+$) $CD8^+$ T cells were found,

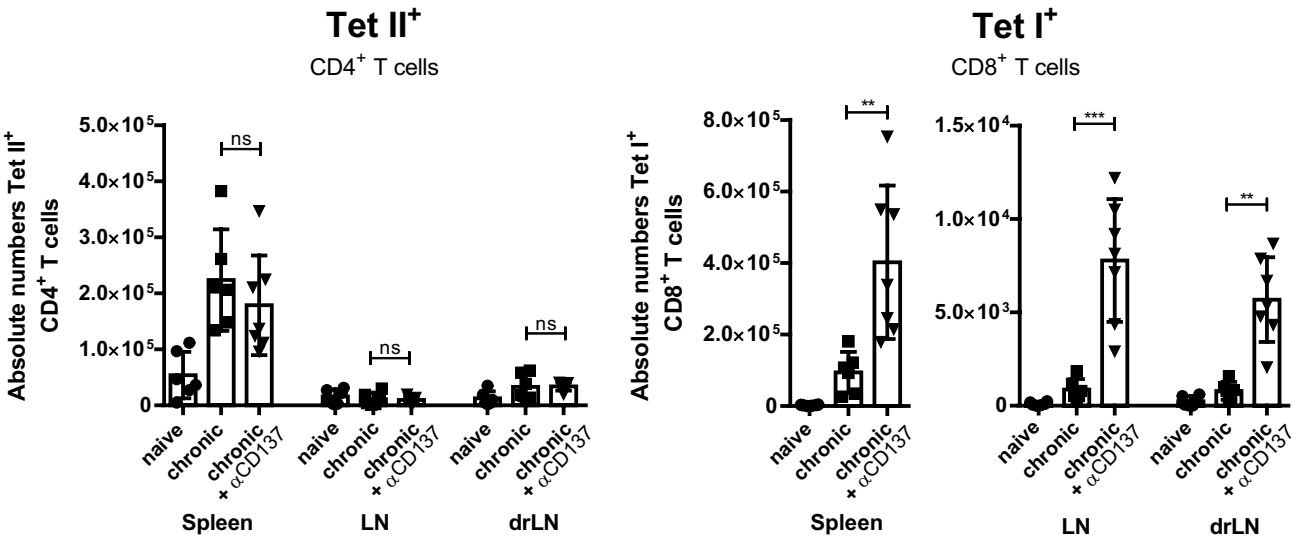


Fig. 2 Influence of αCD137 treatment on the expansion of FV-specific T cells. C57BL/6 mice chronically infected with FV were injected s.c. with 1×10^7 FBL-3 cells and treated with αCD137 antibody. Absolute numbers of Tet II$^+$ CD4$^+$ T cells and Tet I$^+$ CD8$^+$ T cells specific for immunodominant FV epitopes were determined in different organs. Statistically significant differences between the groups were analyzed by the unpaired *t* test: $^{**}p<0.005$; $^{***}p<0.0001$; *ns* not significant. Data were pooled from two independent experiments

and a population of effector phenotype (CD43$^+$CD62L$^-$, [27]) CD4$^+$ T cells with Eomes expression appeared.

Eomes drives the expression of the apoptosis-inducing molecule granzyme B (GzmB), so we investigated if the αCD137 treatment augments GzmB production. Again, virtually no GzmB expression was found in activated CD4$^+$ T cells from chronically FV-infected mice and the percentages of GzmB$^+$ CD8$^+$ T cells were very low as described before (Fig. 3b, Suppl Fig. 1a and [28]). αCD137 treatment significantly augmented the percentages of both GzmB-producing activated CD4$^+$ and CD8$^+$ T lymphocytes, measured without in vivo re-stimulation in direct ex vivo staining. Only in lymph nodes, this difference was not statistically significant for CD8$^+$ T cells.

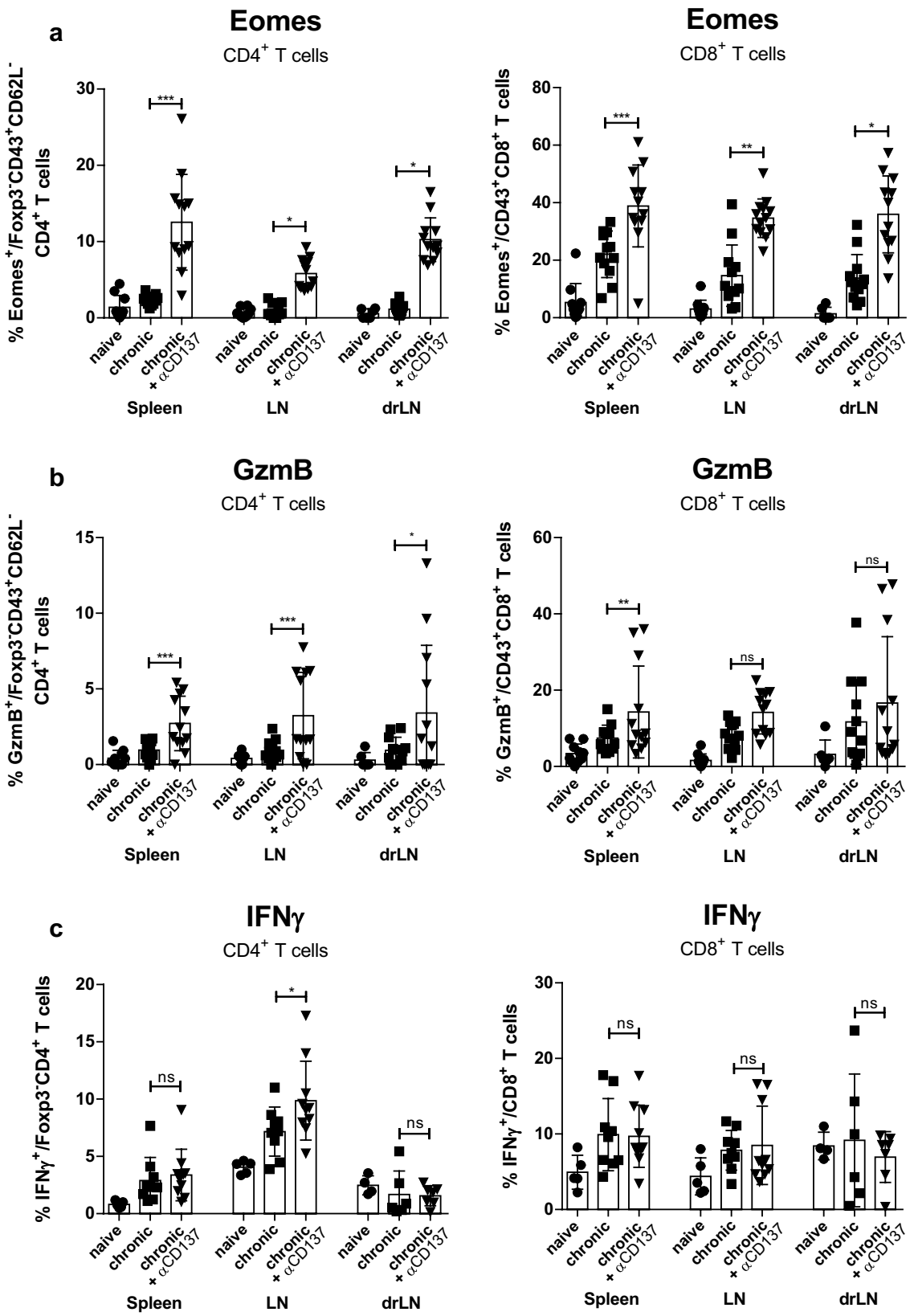
Interestingly, while the frequencies of GzmB$^+$ T cells were strongly increased by αCD137 therapy, GzmB expression levels per cell (MFI) were unchanged, suggesting that GzmB expression was not just enhanced but newly induced in most T cells (Suppl Fig. 1b). Different results were obtained for IFNγ expression after αCD137 therapy. The percentages of IFNγ-producing activated CD4$^+$ and CD8$^+$ T cells were largely unchanged after the administration of αCD137 into chronically infected mice, with the only exception of CD4$^+$ T cells in lymph nodes (Fig. 3c).

Taken together, FBL-3 tumor rejection through CD137 agonistic signaling in chronic FV infection correlated with the reactivation of the cytotoxic pathway in dysfunctional CD8$^+$ T cells and the induction of cytotoxicity in CD4$^+$ T cells.

An in vivo cytotoxicity assay reveals FBL-3 tumor cell killing after CD137 agonistic therapy

Our next intention was to determine whether these cytotoxic T cells contribute to tumor cell killing or not. To answer this question, we performed a series of in vivo killing experiments, in which we used FV-induced FBL-3 tumor cells as targets in FV-infected αCD137-treated mice. As a non-FV-related control, we used the chemically induced lymphoma EL-4 cell line [29]. In mice that were chronically FV-infected but untreated, killing of target cells was around the unspecific detection limit of 10% (Fig. 4a), confirming that chronically infected mice show impaired T cell cytotoxicity [21, 30]. FBL-3 killing was significantly higher in the αCD137-treated mice (around 25%), detected at 4 hs after intraperitoneal cell transfer. All treated mice were depleted for NK cells during the assay to exclude NK cell-mediated tumor killing. Thus, in response to αCD137 therapy, T cells acquired an effector phenotype facilitating FBL-3 cell elimination. Since FBL-3 cells express both CD4$^+$ and CD8$^+$ T cell epitope antigens, the assay cannot distinguish between CD4$^+$ or CD8$^+$ T cell killing.

Hence, we determined the T cell population(s) responsible for the anti-tumor response upon costimulatory treatment. Therefore, we separately depleted FV-infected αCD137-treated mice for their CD4$^+$ or CD8$^+$ T cells and challenged them with FBL-3 cells. Depletion of CD4$^+$ T cells resulted in tumor progression in the first 2 weeks after FBL-3 challenge. Similarly, CD8$^+$ T cell depletion


a
Eomes
CD4+ T cells
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Eomes
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CD8+ T cells
% GzmB+/CD43+CD8+ T cells
c
IFNγ
CD4+ T cells
% IFNγ+/Foxp3-CD4+ T cells
IFNγ
CD8+ T cells
% IFNγ+/CD8+ T cells
naive
chronic
chronic + αCD137
Spleen
LN
drLN
ns

◂**Fig. 3** αCD137 treatment activates cytotoxic $CD4^+$ and $CD8^+$ T cell populations. C57BL/6 mice chronically infected with FV were injected s.c. with 1×10^7 FBL-3 cells and treated with αCD137 antibody. **a** Frequencies of $CD4^+$ and $CD8^+$ T cells expressing the intracellular transcriptional factor Eomes. **b** Frequencies of $CD4^+$ and $CD8^+$ T cells expressing GzmB. **c** Frequencies of $CD4^+$ and $CD8^+$ T cells expressing IFNγ. Statistically significant differences between the groups were determined by the unpaired *t* test: $*p < 0.05$; $**p < 0.005$; $***p < 0.0001$; ns, not significant. *LN* lymph nodes, *drLN* tumor-draining lymph node. Data were pooled from three independent experiments

also led to tumor growth (Fig. 4b). Thus, our data demonstrate that costimulatory CD137 therapy in chronic FV infection augments anti-tumor cytotoxic $CD4^+$ and $CD8^+$ T cell responses, which both significantly contribute to tumor control.

Discussion

Despite the fact that the use of costimulatory antibodies in clinical practice has dramatically increased in the past years, the field of antibody-based tumor therapy deserves more precise investigation as its immunomodulatory mechanisms are not completely understood [31]. The efficacy of the CD137 agonistic antibody had been described in different tumor models since it was first identified as a possible target for anti-tumor immunotherapy [17, 32, 33]. After multiple promising animal studies, the results of αCD137 costimulation in clinical therapy were largely disappointing, since Urelumab, the first αCD137 monoclonal antibody in clinical trials, revealed dose-dependent hepatotoxicity [34]. In addition, Utomilumab, another CD137 agonist, was less potent than Urelumab and mediated only low immune cell activation [35]. Thus, improved therapeutic antibodies against CD137 are currently under development.

Moreover, it remained unclear if the promising anti-tumor effects of αCD137 could also be found in chronically infected individuals. Therefore, it was crucial for our experiments to use chronically FV-infected mice because tumor immune surveillance in the context of a chronic infection is a largely understudied subject. In this work, we show that the additional costimulatory signal, provided by αCD137, but not αCD134 therapy, could restore tumor immune responses in chronically FV-infected mice. We also demonstrate that effector $CD8^+$ and $CD4^+$ T cells can gain anti-tumor activity after CD137 costimulatory immunotherapy. Interestingly, this tumor rejection in chronically FV-infected mice required the interplay of both $CD8^+$ and $CD4^+$ T lymphocytes and was abrogated in either $CD8^+$ or $CD4^+$ T cell-depleted mice. T cell cytotoxicity was dependent on the induction of the transcription factor Eomes that can trigger the cytotoxic program in T cells, including GzmB expression and tumor cell killing. However, the molecular mechanisms underlying the αCD137-mediated cytotoxicity might be quite different between $CD4^+$ and $CD8^+$ T cells. FBL-3 tumor challenge, as well as FV infection of naïve C57BL/6 mice, induces strong cytotoxic $CD8^+$ T cell responses [36], which then become exhausted during the development of chronic FV infection [30]. Thus, the cytotoxicity of $CD8^+$ T cells after αCD137 therapy is most likely a reactivation of this exhausted $CD8^+$ T cell pool. Studies with chronically FV-infected mice reveal that such a reactivation of $CD8^+$ cytotoxicity is possible [37]. Our study also indicates that numbers of FV-specific $CD8^+$ T cells increase after αCD137 therapy, suggesting a proliferation of the specific cells due to the co-stimulation. However, this does not exclude the possibility that therapy-activated T cells, which contribute to tumor rejection, might also recognize other tumor antigens than we stained for with our tetramers.

The situation for $CD4^+$ T cells is different since acute FV infection, as well as FBL-3 inoculation per se, does not induce the cytotoxic program in $CD4^+$ T cells [3, 38]. Thus, the effect of αCD137 therapy seems to be a de novo induction of cytotoxic $CD4^+$ T cells in FV-infected tumor-challenged mice. This seems to be a special feature of CD137 signaling, but not CD134 signaling, and we previously showed that αCD137 activation can even convert Tregs into cytotoxic T cells with anti-tumor activity [10].

Interestingly, despite the fact that $CD8^+$ T cells, which had been previously demonstrated to be the major player in the control of FBL-3 tumor growth [39], the cytotoxic $CD4^+$ T cell population contributed significantly to the tumor cell rejection in the setting of chronic infection. This suggests that the expansion and reactivation of $CD8^+$ T cells are not sufficient to mediate tumor control in a chronically infected host. However, the augmentation of the $CD4^+$ T cell response was based on a qualitative improvement, since the numbers of Tet II^+ $CD4^+$ T cells remain unchanged after αCD137 therapy. Again, we can only conclude that for one T cell epitope, and $CD4^+$ T cells with other specificities may be involved in a successful therapy.

It has been demonstrated that triggering CD134, also known as OX40, improves anti-tumor immunity in combination with different anti-cancer agents and decreases recurrence rates after surgical therapy of cancer [20, 40]. It is known that CD134 is transiently expressed on conventional T cells and constantly on Tregs. This is supported by the findings that anti-tumor effects following CD134 costimulatory therapy can be mediated not only by $CD8^+$ and conventional $CD4^+$ T cells, but also via Treg manipulation [31]. However, stimulation of Tregs can result in different outcomes in the context of progression and spread of neoplastic cells [41] and in our model it counter-regulated the beneficial effects.

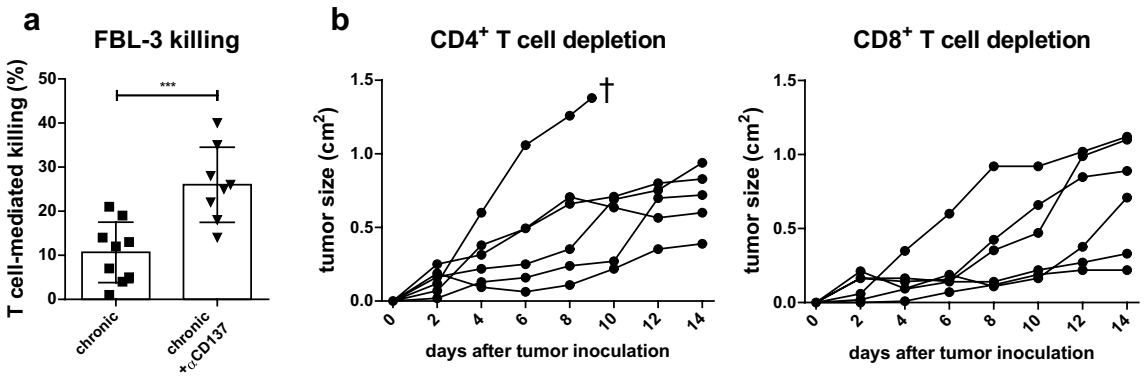

Fig. 4 In vivo tumor cell killing by T cells after αCD137 therapy and the influence of different T cell populations on tumor rejection. C57BL/6 mice chronically infected with FV were injected s.c. with 1×10^7 FBL-3 cells and treated with αCD137 antibody. **a** Frequencies of T cell-mediated killing of FBL-3 cells in an in vivo cytotoxicity assay (described in "Materials and methods"). One day prior to the experiment, mice from both groups as well as naïve ones were depleted for NK cells to focus on T cell killing. Statistically significant differences between the groups were determined by the unpaired *t* test: $*p<0.05$; $**p<0.005$; $***p<0.0001$; ns, not significant. Data were pooled from two independent experiments. **b** Mice were depleted for either $CD4^+$ or $CD8^+$ T cells and treated with αCD137 as described in "Materials and methods". Each line represents tumor progression in an individual mouse. †Mouse was euthanized because of progressive tumor growth

One question concerning the successful CD137 therapy was if the therapy affects the chronic FV infection, and if it is critical for the recovery of tumor immunity. In our previous report, we showed that the administration of CD137 antibody was associated with a reduction in chronic FV loads, but did not eliminate the virus completely [21]. Reduced viral loads might be beneficial for the reactivation/induction of anti-tumor T cell cytotoxicity, but the αCD137 therapy did not result in complete viral clearance, so the overall significance for the antitumor immunity remains questionable.

In conclusion, in this work, we propose that αCD137 agonistic therapy during the chronic phase of FV infection can restore anti-FBL-3 tumor control mediated by $CD8^+$ and $CD4^+$ T cells. Thus, agonistic antibodies to CD137 can efficiently enhance anti-tumor immunity even in the setting of chronic viral infection, which might have promising therapeutic applications.

Author contributions Anna Malyshkina and Ulf Dittmer conceived the presented study and wrote the manuscript. Anna Malyshkina and Elisabeth Littwitz-Salomon carried out the experiments and analyzed data. Anna Malyshkina, Elisabeth Littwitz-Salomon, Sonja Windmann, Jean Alexander Ross, and Simone Schimmer were involved in the sample preparation. Kathrin Sutter and Annette Paschen contributed to the interpretation of the results. Jean Alexander Ross assisted with the design of the figures. All authors discussed the results, provided critical feedback and contributed to the final manuscript.

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Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV) (Permit number: G 1518/15). All efforts were made to minimize suffering.

Animal source Female C57BL/6 mice between 6 and 10 weeks old were purchased from Envigo, Germany.

Cell line authentication The FBL-3 cell line is a Friend virus-induced leukemia cell line, generated in a C57BL/6 mouse. The EL-4 cell line is a chemically induced lymphoma cell line, generated in a C57BL/6 mouse by 9,10-dimethyl-1,2-benzanthracene. Both cell lines were a gift from Kim J. Hasenkrug (Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA). They were expanded, aliquoted, and frozen for further use. Once in culture, cells were not continuously passaged. The identity of the cell lines was confirmed by biological assays. Before the experiments, cell lines were tested in naïve C57BL/6 mice: FBL-3 cells inoculated into the right flank of the mice were rejected, whereas EL-4 cells were not.

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