Control of regulatory T cells is necessary for vaccine-like effects of antiviral immunotherapy by monoclonal antibodies

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Abbreviations: mAb: monoclonal antibody; ADCC: antibody-directed cell-mediated cytotoxicity; CDC: complement-dependent cytolysis; FIA: focal immunofluorescence assay; Env: envelope glycoprotein; FFU: focus-forming units; MuLV: murine leukemia virus, FV: Friend retroviral complex.
Regulatory T cells (Tregs) down-regulate immunity and are associated with chronic viral infections, suggesting that their inhibition might be used to treat life-threatening diseases. Using the FrCasE mouse retroviral model, we have recently shown that short monoclonal antibody-(mAb)-based immunotherapies can induce life-long, protective immunity. This has potentially important therapeutical impact, as mAbs are increasingly considered to treat severe viral infections. We now report that poor anti-FrCasE immunity in infected mice is due to Treg expansion in secondary lymphoid organs, as depletion of Tregs restores humoral and CTL antiviral responses. Kinetic analyses show that Treg expansion is not a consequence of chronicity but is associated with viral spread. Moreover, Treg adoptive transfers indicate that production of the immunosuppressive cytokine IL-10 is essential for preventing a protective immune response. Finally, treatment of infected mice with a virus-neutralizing IgG2a, shortly after infection, prevents Treg expansion and limits immunosuppressive activity. This effect is rapid, necessary for the development of protective immunity and depends on mAb effector functions. Manipulating Tregs may, therefore, be necessary to confer robust antiviral immunity in the context of mAb-based therapy. This concept likely applies to cancer treatment, as vaccine-like effects of mAbs were also observed in certain cancer immunotherapies.
INTRODUCTION

Regulatory T cells (Tregs) are a subset of CD4\(^+\) T lymphocytes downregulating the immune system. As such, they are key modulators of the establishment and/or maintenance of viral chronicity and constitute a barrier to efficient vaccination and immunotherapy strategies. The implication of Tregs in chronic viral infection was first described in mice infected with the Friend virus complex (FV) and then extended to other persistent viruses, including HIV, HBV, HCV, EBV, LCMV, HSV and HPVs. It is increasingly clear that controlling this immunosuppressive cell subset would have widespread clinical applications to fight life-threatening viral diseases.

Monoclonal antibodies (mAbs) constitute the largest class of biotherapeutics. Their main current applications concern cancer and inflammatory diseases. Moreover, they have an enormous potential to treat both acute and chronic severe viral infections. Although the first antiviral mAb to be commercialized, in 1998, was an anti-RSV to treat an infant respiratory disease, many of the current antiviral mAbs have been developed more recently. Illustrating this trend, humanized mAbs targeting diverse viruses (Ebola, WNV, CMV, human and avian influenza, SARS, Hanta and Nipah) responsible for acute diseases have recently shown promising results in preclinical animal settings. Some of them are now undergoing clinical trials. Many chronic infections have also been targeted. Thus, several human anti-HIV mAbs have been tested in non-human primates (see), as well as in clinical trials, and broadly neutralizing human anti-HIV mAbs have recently been isolated. Several human anti-HCV mAbs have also recently been developed, some of them currently being tested in the clinic.

The direct effects of mAbs (inhibition of soluble effectors or of cell membrane signaling receptors, virus neutralization, NK-(ADCC) or complement (CDC)-dependent cytolysis…) on their targets are, in general, well studied. Additionally, we have recently shown that short neutralizing mAb-based immunotherapies may exert immunomodulatory effects translating into life-long immune protection (see below), which has potentially important consequences for treating human, life-threatening viral diseases. The recent demonstration that passive neutralizing mAbs could, not only control viremia in young macaques infected with a hybrid simian HIV strain, but also enhance B cell responses in a perinatal setting
further supports this concept. Similar vaccine-like effects were also recently documented in two cancer models immunotherapy-treated with mAbs directed to tumor cell membrane proteins \(^{25,26}\). This emphasizes that immunomodulatory actions of mAbs may find applications in a wide array of pathologies with both a direct therapeutical benefit for patients and an indirect one for the society: mAb-based therapies are expensive, irrespective of the disease concerned, and the capacity to induce long-lasting immune protection by short treatments would considerably reduce their costs. Elucidating the cellular and molecular mechanisms underlying the reinforcement of endogenous immunity of mAb-treated patients will be a major step towards improved immunotherapies.

To test the concept that short immunotherapies with antiviral mAbs can trigger long-lasting protective immunity, we resorted to the FrCas\(^{E}\) mouse simple retrovirus model due to its multiple advantages: (i) the manifestations of pathological effects are rapid with a 100\% incidence and a clear symptomology (fatal neurodegeneration within 2 months if infection occurs before day 5-6 after birth or fatal erythroleukemia within 4-5 months if infection occurs on day 8) (see \(^{20,24}\)), (ii) it is one of the rare chronic viral infection systems for which neutralizing mAbs of the same species are available, permitting investigations in immunocompetent organisms, (iii) mice offer immunological tools that are not available in other species and (iv) infection by FrCas\(^{E}\) is reminiscent of perinatal infection by HIV, which affects 2.5 million children living with HIV infection worldwide (2009 estimate by the World Health Organization. See \(^{22,24}\)). When pups infected perinatally with FrCas\(^{E}\) are treated for a few days with a neutralizing IgG2a/k mAb (667) recognizing the receptor-binding domain of FrCas\(^{E}\) Env glycoprotein within 2 days post-infection, infected/treated mice survive >1 year with no pathological symptoms \(^{20,24}\). Healthy survival depends on a strong protective endogenous immunity, as breaking it entails leukemic death \(^{21}\). This immunity contrasts with the weak response developing in infected/non-treated mice and is of the Th1 type with strong (IgG2a) humoral \(^{20,22,24}\) and a CTL \(^{21,24}\) arms and strong memory component \(^{21,24}\) permitting mice to resist a viral challenge long after primary infection \(^{20}\). Induction of such an immunity does not result from the simple reduction of viremia during immunotherapy affording the immune system more time to react. Instead, it is due to genuine immunomodulatory actions of the mAb that depend on its ability to interact with receptors for IgGs (Fc\(\gamma\)Rs) \(^{23,24}\), in particular on dendritic cells (DCs) that are at the heart of any adaptive immune response. Specifically, interactions between DC Fc\(\gamma\)Rs and immune complexes (IC) comprising the
therapeutic antibody bound to Env on FrCasE infected cells increases uptake of the latter cells by DCs, permits more efficient activation of DCs and is followed by stronger anti-FrCasE CD8+ T-cell responses. Cellular IC/FcγRs interactions are, however, not limited to DCs but also concern NKs that are responsible for ADCC of infected cells during immunotherapy. In contrast, CDC has no detectable role in infected cell lysis.

As chronic viral infections are associated with Treg responses dampening antiviral immune responses, we asked whether the antiviral vaccine-like effects induced by mAb-based immunotherapies could involve an inhibition of Tregs. Here, we characterize the Treg response induced by FrCasE and establish its role in the poor immunity mounted by infected mice. We also report that the 667 treatment inhibits the development of Tregs and that this control is necessary for the mounting of protective immunity in infected, 667 mAb-treated mice. This finding may have important consequences for the design of successful antiviral and anti-cancer immunotherapies based on the use of mAbs.
MATERIALS AND METHODS

Virus stocks. Production, assay and storage of FrCasE stocks were described previously 20-24,27.

Production of 667 and 672 monoclonal antibodies and of the 667 F(ab')2 fragment. The neutralizing mouse mAb 667 (IgG2a/κ) was purified and assayed as previously described 21,29. The mouse mAb 672 (IgM/κ) was prepared from hybridoma cell-culture supernatant and purified using the IgM purification kit from Pierce 23. The 667 F(ab')2 fragment was prepared by proteolysis of 667 with pepsin. The Fc fragment was removed by protein A chromatography and the F(ab')2 fragment was collected in the unbound fraction and quality-controlled as described in ref 23.

Viral infection, immunotherapy and mice follow-up. We used inbred 129/Sv/Ev mice (H-2Db haplotype). Infections were performed intraperitoneally (i.p.) 3 days after birth with 1-5 FFU (low inoculum infections) as previously described 23. Under these conditions, mice develop leukemia with kinetics similar to those of mice infected with 5 x 10^4 FFU (high inoculum infection) on day 8 after birth (see ref 24). Treatments with the 667 and 672 mAbs or the 667 F(ab')2 fragment consisted of repeated i.p. administrations (15 µg of antibody each time), starting 1-2 hours post-FrCasE inoculation (which is sufficient for establishment of infection), as previously described 20-24. Mice were examined daily for clinical signs of neurodegeneration or erythroleukemia (see 20-24 for details). Plasma anti-FrCasE serum immunoglobulins (Ig) were assayed by ELISA as already described 20,27. For challenge experiments, mice were injected intravenously with a mix containing 25 µl of FrCasE suspension (5 x 10^5 FFU/ml) and 1 x 10^6 infected fresh splenocytes.

Flow cytometry analyses. They were conducted using the FACSCanto II device (Becton Dickinson). The data were processed using the FlowJo program (Three Stars). FrCasE-infected cells were assayed using an anti-Env mAb labelled with FITC (see ref. 23,24). Staining of cell surface markers (CD4, CD8, CD25, CTLA4, GITR, Helios, CD39) and intracellular proteins (granzyme B, FoxP3, IFN-γ, IL10, TGF-β) were performed using BD Biosciences Pharmingen-, e-Bioscience or BioLegend antibodies, which were either, FITC-, APC-Alexa-, PE- or PE-Cy7-conjugated. Granzyme B, FoxP3, IFN-γ, IL-10 and TGF-β using the Cytofix/Cytoperm intracellular staining kit (Becton Dickinson).
**In vivo depletion of CD4⁺CD25⁺ T cells.** The anti-CD25 IL-2Rα mAb produced (PC61 hybridoma; BioXcell) was used for *in vivo* depletion of CD25⁺ T cells, as described by others. Infected/non-treated mice received 4 injections (25 μg each) of this mAb on days 2, 4, 7 and 9 post-challenge. Depletion of Tregs was monitored by flow cytometry of CD25<sup>high</sup>FoxP3⁺CD4⁺ T cells.

**Purification and adoptive transfer of Treg cells.** CD4⁺CD25⁺ T cells were isolated from splenocytes of donor mice using the MACS CD4⁺CD25⁺ regulatory T cell magnetic isolation kit (Miltenyi Biotech). Cell purity (>95%) was verified by flow cytometry using FoxP3 staining. For adoptive transfer, 3 × 10⁵ purified cells in 0.1 ml of PBS were tail vein-injected into recipient mice.

**In vitro T-cell activation.** 5 x 10⁵ splenocytes were incubated at 37°C for 5 h in 96-well round-bottom plates in 200 μl of RPMI culture medium containing 5 μg/ml brefeldin A (Sigma-Aldrich). T-cell activation was assessed in IFN-γ flow cytometry assays using PMA (50 ng/ml) + ionomycin (500 ng/ml)-stimulated splenocytes as positive controls and untreated cells as negative controls.

**In vitro Treg activation.** 5 x 10⁵ Tregs purified as described above were incubated at 37°C for 72 h in 96-well round-bottom plates in 200 μl of RPMI containing 1 mM Na-pyruvate (GIBCO-100mM), 1X non-essential amino acids (GIBCO-MEM NEAA 100X), 50 μM β-mercaptoethanol (GIBCO-50mM), 20 ng/ml recombinant mouse IL-2 (Sigma-Aldrich) and 0.5 μg/ml anti-CD28 antibody (Becton Dickinson). For TCR stimulation experiments, wells were precoated at 37°C for 2 hours with an anti-CD3 mAb (Becton Dickinson) using a 1 μg/ml solution in bicarbonate buffer pH 8.8. 5 μg/ml Brefeldin A (Sigma-Aldrich) was added 5 hours before labeling for flow cytometry analysis.

**Treatment of mice with the anti-IL10 mAb.** Mice were injected intraperitoneally once a day using 0.5 mg of the anti-IL-10 JES5–2A5 mAb (BioXcell) from day 5 (day of Treg grafting) to day 10 post-viral challenge (day of sacrifice), as detailed above.

**In vivo cytotoxicity assays.** The experiments were conducted as described in refs. Treatment of mice with the anti-IL10 mAb. Mice were injected intraperitoneally once a day using 0.5 mg of the anti-IL-10 JES5–2A5 mAb (BioXcell) from day 5 (day of Treg grafting) to day 10 post-viral challenge (day of sacrifice), as detailed above.

**In vivo cytotoxicity assays.** The experiments were conducted as described in refs. 21,24. Spleens were recovered from 10 day-old FrCas<sup>E</sup>-infected or non-infected pups. Splenocytes
from non-infected mice were labeled with the vital dye carboxy-fluorescein succinimidyl ester (CFSE; Molecular Probes) at a concentration of 0.5 µM (CFSE\textsuperscript{low} cells) whereas splenocytes from infected mice were labeled in a 5 µM CFSE solution (CFSE\textsuperscript{high} cells). The two cell populations were mixed in a 1:1 ratio before intravenous administration to recipient mice. The ratio of CFSE\textsuperscript{low}/CFSE\textsuperscript{high} cells in spleen was assayed by flow cytometry 24 hours later and the CTL activity against infected splenocytes was calculated from the ratio of CFSE\textsuperscript{high}/CFSE\textsuperscript{low} cells as described in refs. \textsuperscript{21,24}.

**Flow cytometry assay of CD8\textsuperscript{+} T cells specific for FrCas\textsuperscript{E}-infected cells.** Splenocytes were labeled with both an APC-conjugated anti-CD8\textsuperscript{+} T cell antibody and a PE-conjugated MHC class I H-2D\textsuperscript{b} tetramer (Beckman Coulter) displaying the immunodominant Friend virus GagL epitope (D\textsuperscript{b}-GagL tetramers) as previously described \textsuperscript{24}.

**Statistics.** Data are presented as means ± standard deviation. Statistical significance was established using the Student’s t test. A $P$ value of less than 0.05 was considered significant.

**Ethics statement.** All procedures for animal handling and experiments were approved by the local animal facility “ComEth” Institutional Review Board under the supervision of the French LR Regional CEEA ethic committee on animal experimentation.
RESULTS

Treg response induced upon FrCasE infection
We first asked whether FrCasE induced a Treg response associating with chronicity after neonatal infection. For this, we compared Treg abundances in spleens of control (non-infected/non-treated) and perinatally infected (infected/non-treated) mice of 8 weeks of age using classical flow cytometry parameters for Tregs: positivity for both CD4 and the FoxP3 transcription factor and expression of high levels of CD25 (IL-2 receptor α chain) (also see below). CD4⁺CD25highFoxP3⁺ Tregs were much more abundant in adult infected/non-treated mice (14.9 ± 3.64% of total CD4⁺ cells) (Figures 1A-B) than in adult non-infected/non-treated mice (2.4 ± 0.3% of total CD4⁺ cells) (Figures 1A-B), showing induction of a Treg response following infection by FrCasE.

The Treg response induced by FrCasE is inhibited by the 667-mAb immunotherapy
Next, we addressed whether the induction of the Tregs in infected/non-treated mice could be inhibited upon 667 mAb treatment. This was tested in the spleen of 8 week-old animals that were 667-treated shortly after infection (infected/667-treated mice). Tregs levels at adult age were dramatically reduced in infected/667-treated mice as compared to infected/non-treated animals (Figures 1A-B). Then, infected mice were treated with either the 672 mAb (infected/672-treated) or the 667 F(ab')2 (infected/667-F(ab')2-treated). 672 is a neutralizing IgM/κ recognizing an epitope contained within that of 667. It displays no binding activity for FcγRs, but shows high binding activity for complement, whereas the 667 F(ab')2 is devoid of any effector function. None of these treatments inhibited the induction of Tregs (17.2 ± 2.82% and 20.44 ± 1.22% at week 8, respectively) (Figures 1A-B). Thus, the vaccine-like effect of the 667 immunotherapy associated with an inhibition of Treg expansion by FrCasE that depended on the presence of the Fc part of the administered mAb. The effector function involved was most probably binding to FcγR, as shown in former work and here by the fact that neither the 667 F(ab')2 nor the 672 mAb has an effect despite the high complement-binding activity of 672.

Inhibition of Treg expansion by 667 is a rapid event
Then, we asked when inhibition of Treg expansion by the 667 immunotherapy occurred. First, we compared Treg appearance kinetics in non-infected/non-treated and infected/non-treated
mice from day 7 to day 35 post-infection in central (thymus) and peripheral lymphoid organs (lymph nodes and spleen) where MLVs propagate (Figure 1C). In control non-infected/non-treated mice, the fraction of Tregs amongst total CD4+ T cells steadily increased in the thymus throughout the analysis period and remained nearly constant in spleen and lymph nodes (Figure 1C), consitently with central immune system development. In the thymus of infected/non-treated mice, Tregs levels increased moderately (50%) and represented a small fraction (1.2%) of total CD4+ cells (Figure 1C). In contrast, they dramatically increased in spleen and lymph nodes (3 and 4-fold, respectively) where they represented a large fraction of CD4+ T cells (12 and 10%, respectively) (Figure 1C). In all 3 organs, differences with non-infected/non-treated mice appeared rapidly, i.e. as early as day 9 post-infection. Interestingly, Treg expansion was associated with viral spread in infected/non-treated mice, as shown here by flow cytometry of retroviral Env-expressing cells in thymus, spleen and lymph nodes (Figure 1D) and, in former work (see ref. 23), by assay of plasma viremia. Specifically, Treg levels reached a maximum by days 14-19 depending on the organ and remained relatively stable for the period of analysis, which correlated with high levels of cell infection in the 3 organs. Finally, an increase in Tregs was observed in spleen and lymph nodes, but not in thymus, when infected/non-treated mice were virally challenged on week 8 post-infection (Figure 1E), raising the possibility of antigen-dependent induction of Treg proliferation in secondary lymphoid organs. Strikingly, no detectable induction of Tregs was seen in thymus, spleen and lymph nodes of infected/667-treated animals from day 7 to day 35 post-infection (Figure 1C). This contrasted with infected/672-treated- and infected/667-F(ab')2-treated mice behaving as infected/non-treated ones (Supplementary Figure 1). Thus, inhibition of Treg expansion by 667 immunotherapy was a rapid, if not an immediate, event.

**Treg response in FrCasE-infected mice correlates with poor CTL activation**

We then asked if Tregs in infected/non-treated mice contributed to infection chronicity and if lack of Treg expansion in infected/667-treated animals was necessary for the generation of protective immunity. Having previously shown that the number of CTLs specific for FrCasE-infected cells was much lower in infected/non-treated- than in infected/667-treated mice 21,24, we first assessed whether the levels of Tregs and the activation state of CTLs could be inversely correlated between these two groups of animals. The number of CTLs specific to infected cells being too low in infected/non-treated mice to conduct a rigorous analysis, we compared total splenic CD8+ T cells positive for the functional markers IFN-γ and granzyme B (GzmB) in different groups of mice 8 weeks after infection. CD8+IFNγ+ and CD8+GzmB+
cell numbers were very low in non-infected/non-treated and infected/non-treated mice compared to infected/667-treated ones (Figures 2A-B). Consistently with a role for 667 effector functions in protective immunity induction, results for infected/672-treated- and infected/667-F(ab')2-treated mice were similar to those of infected/non-treated animals. Thus, increased levels of Tregs, not only associated with lower levels of CTLs directed to FrCasE-infected cells, but also with poorer CTL activation. Next, we asked whether these features could be associated with increased levels of PD-1, as this marker is, most often, a negative regulator expressed at the surface of CD8+ T lymphocytes of which it promotes apoptosis and exhaustion 29. This was achieved at different times points after infection in spleen and lymph nodes (Figure 2C-D), as these organs showed the highest levels of induced Tregs in infected/non-treated mice (Figure 1C). Basal levels of PD-1+CD8+ T cells remained comparable in non-infected/non-treated- and infected/667-treated animals for the whole experiment duration. In contrast, PD-1 was strongly induced in infected/non-treated mice with accumulation kinetics comparable to those of Tregs up to day 19 (at which time Treg numbers have already reached their plateau). Notably, infected/672-treated and infected/667-F(ab')2-treated mice behave like infected/non-treated mice (Supplementary Figure 2). Thus, PD-1 expression levels inversely correlated with the number of CD8+ T cells expressing IFNγ and GzmB in the various groups of animals analyzed, raising the possibility that Tregs exerted their effects on anti-FrCasE CTLs, at least in part, via the induction of PD-1 in infected/non-treated mice (see Discussion).

**Tregs repress both cellular and humoral anti-FrCasE immune responses in infected/non-treated mice**

Next, we assessed the functional role of Tregs in the poor anti-FrCasE humoral and CTL responses in infected/non-treated mice. To this aim, mice were infected perinatally and re-challenged at 8 weeks of age before analysis of both types of responses (see Figure 3A) in the presence or in the absence (Figure 3B) of Tregs, which were depleted via repeated intraperitoneal administration of an anti-CD25 mAb (96% efficiency), as previously described by others 28. Anti-FrCasE humoral response was 5-fold more elevated in Treg-depleted mice versus control animals (Figure 3C), predominantly of the IgG2a isotype and as neutralizing as that found in infected/667-treated mice of the same age (not shown). Notably, Treg depletion restored humoral immunity in both infected/672-treated and infected/667-F(ab')2-treated mice with endogenous IgG levels comparable to those observed in infected/667-treated mice (not shown). Anti-FrCasE CTL immunity being dependent on the
immunodominant GagL epitope derived from the retroviral Gag protein precursor, we used a fluorescent D<sup>b</sup>-GagL tetramer to monitor it by flow cytometry (see 21,24 for references). Treg depletion induced an 8-fold increase in the percentages of D<sup>b</sup>-GagL tetramer<sup>+</sup>CD8<sup>+</sup> splenic cells (Figures 3D-E). Moreover, an in vivo cell-killing assay (see Materials and Methods and Legend to Figure 3) showed that CTL activity against FrCas<sub>E</sub>-infected cells was 7-fold higher in Treg-depleted mice (Figures 3F-G). Consistently with the role of 667 Fc<sub>γ</sub>R-binding effector function in the induction of protective immunity 23,24, infected/667-F(ab')<sub>2</sub>-treated- and infected/672-treated mice behave similarly to infected/non-treated animals concerning both humoral and CTL responses (not shown). Thus, Tregs induced by FrCas<sub>E</sub> in infected/non-treated mice inhibited the mounting of efficient antiviral humoral and cellular responses.

The absence of induced Tregs is necessary for protective immunity induced by 667 immunotherapy

We then addressed whether the absence of induced Tregs in infected/667-treated mice was necessary for the induction of protective immunity by asking whether Tregs from infected/non-treated mice could disrupt their anti-FrCas<sub>E</sub> immunity (See Figure 4A for experimental scheme). Highly enriched Treg populations from 8 week-old infected/non-treated (78 ± 5% of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells) and non-infected/non-treated mice (84 ± 3% of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells) were prepared and administered to infected/667-treated mice of the same age, which were virally challenged 5 days earlier to optimize the analysis of humoral and CTL immune responses. In contrast to Tregs originating from non-infected/non-treated animals that had only a moderate effect, those from infected/non-treated mice reduced (i) the number of CTLs specific for FrCas<sub>E</sub>-infected cells (Figure 4B), (ii) CTL activity against FrCas<sub>E</sub>-infected cells (Figure 4C) and (iii) humoral immunity against FrCas<sub>E</sub> (Figure 4D). Notably, CTLs from infected/667-treated mice receiving Tregs from infected/non-treated animals also had reduced levels of IFNγ and GzmB (not shown). This was reminiscent of what happened in infected/non-treated mice where high Treg levels were associated with, not only reduced levels of CTLs directed to infected cells, but also with a reduction in their activation state (see Figures 2C-D). Underlining functional differences between Tregs from infected/non-treated- and infected/667-treated mice, infected/667-treated mice grafted with Tregs purified from other infected/667-treated animals of the same age (82 ± 6% of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells), behave as those grafted with control Tregs from non-infected/non-treated mice (Figure 4B-D). Moreover, Tregs, prepared from 8 week-old, infected/667-
F(ab’)2-treated- (79 ± 4 % of CD4+CD25+FoxP3+ cells) or infected/672-treated mice (86 ± 5% of CD4+CD25+FoxP3+ cells) induced a strong and rapid inhibition of endogenous anti-FrCasE immunity developing in infected/667-treated mice (Figure 4B-D). Thus, as none of the differences observed in these experiments was attributable to differences in Treg enrichment, our data indicated that Tregs from infected/non-treated mice and infected/667-treated animals show different functional properties: whereas those induced in infected/non-treated mice by FrCasE exerted strong suppressive activity on anti-FrCasE immunity, those present in infected/667-treated animals were tolerant to this immunity.

**Tregs from infected/non-treated and infected/667-treated show phenotypic differences**

We then phenotyped CD4+CD25+FoxP3+ cells in further detail using well described Treg phenotypical and functional markers (CTLA4, Helios, GITR, CD39 and GzmB) and the intracellular expression of the immunosuppressive cytokines TGF-β and IL-10 to identify functional differences between Tregs from infected/non-treated and infected/667-treated mice. For this, Tregs from 8 week-old non-infected/non-treated-, -infected/non-treated- or -infected/667-treated mice purified from spleen were flow cytometry analyzed after TCR stimulation. No major differences were observed for GITR, CD39, Granzyme B and TGF-β (Figure 5A). In contrast, higher levels of CTLA4, Helios and intracellular IL-10, 3 markers often associated with Treg activation and immunosuppressive activity 29,31, were clearly detected in the case of Tregs from infected/non-treated mice whereas the other two groups of mice showed comparable amounts (Figure 5A).

**IL-10 is key immunosuppressor effector of Tregs in infected/non-treated mice**

The above data prompted us to ask whether IL-10 produced by Tregs could play a key role in the poor immunity mounted by infected/non-treated animals. To this aim, Tregs were purified from infected/non-treated mice and then grafted to 8 week-old infected/667-treated mice, which were virally challenged at the same time as in Figure 4A. Half of the animals were subjected to intraperitoneal administration of a neutralizing anti-mouse IL-10 mAb from day 5 to day 10 post-viral challenge/Treg grafting, at which time anti-IL-10-treated- and -non-treated mice were sacrificied for immune response analysis. Our data showed that (i) the number of CTLs specific for FrCasE-infected cells (Figure 5B), (ii) CTL activity against FrCasE-infected cells (Figure 5C) and (iii) humoral immunity against FrCasE (Figure 5D) were maintained only in the presence of the anti-IL-10 mAb. This suggested that IL-10 was a key immunosuppressive effector of Tregs in infected/non-treated mice.
DISCUSSION

Using the FrCas\textsuperscript{E} model system, we have previously shown that short antiviral mAb-based immunotherapies generate vaccine-like effects characterized by strong and long-lasting antiviral humoral and cytotoxic T-cell responses \textsuperscript{20-24}. This has potentially important therapeutical consequences for future treatment of patients infected with life-threatening chronic viruses. Here, we report that induction of such a long-lasting, antiviral, protective immunity critically relies on inhibition of the Treg response that is otherwise induced upon infection and permits chronicity establishment and pathological manifestations. Our findings underline that manipulating Treg populations represents a necessary condition to confer robust and sustained antiviral immunity via mAb-based immunotherapy.

Most often, it is not fully clear when and how Tregs are generated in chronic infections and even whether they are the cause or the consequence of chronicity, especially in young infected organisms except one report on immune response modulation by Tregs in HIV-exposed infants \textsuperscript{32}. Our herein kinetic analyses in infected/non-treated mice indicate that Treg expansion is rapid, occurs during acute infection and precedes resorbtion of this phase and establishment of FrCas\textsuperscript{E} chronicity. This situation is reminiscent to that of FV \textsuperscript{33,34} (the infection model closest to FrCas\textsuperscript{E}) and HSV neonatal infection where Tregs suppress infant antiviral CTL responses \textsuperscript{35}. Also reminiscent of FV, where induction of Tregs is compartmentalized to sites of inflammation \textsuperscript{36}, FrCas\textsuperscript{E}-induced Tregs principally expand in lymph nodes and spleen where viral replication mainly occurs. Moreover, Tregs induced by FrCas\textsuperscript{E}, not only reduce the number and cytolytic activity of CTLs, but also alter their phenotype. Thus, decreased expressions of IFN\textgamma{} and Granzyme B were observed on CTLs at adult age, as also described in the FV model \textsuperscript{37}, as well as increased levels of PD-1 at the end of the neonatal period. Although the latter observation is consistent with observations by others that PD-1 is expressed on exhausted CTLs in diverse chronic viral infection \textsuperscript{38-40}, it must be noted that an intact PD-1 pathway has also been described to enhance immunity during acute viral infection \textsuperscript{41}. This indicates that its role in infected/non-treated mice still requires further exploration, which is all the more important that PD-1 effects must be considered in combination with other markers of Tregs \textsuperscript{29,42}. 

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Beyond the fact that they highlight similarities and differences between FV- and the FrCas^E retroviral systems, our data exclude that Treg expansion is the consequence of infection chronicity leading to CTL exhaustion and loss of immune memory via continuous antigen stimulation, a possibility that has been raised elsewhere^43. Rather, several lines of evidence strongly suggest that Tregs induced during acute infection contribute to the maintenance of viral chronicity at later times through inhibition of the antiviral immune response. Firstly, Tregs from infected/non-treated mice were more efficient at depressing the immune response of infected/667-treated animals in adoptive transfer experiments than Tregs from non-infected/non-treated- or infected/667 treated mice. Secondly, Tregs from infected/non-treated mice show higher levels of Helios and CTLA-4, which are Treg markers whose high expression is usually associated with activation and suppressive potential^29,31. Thirdly, we show that IL-10, produced by Tregs from infected/non-treated mice, is a key factor for these cells to exert an immunosuppressive action upon grafting in infected/667-treated mice. Finally, depletion of Tregs in infected/non-treated mice led to the restoration of a strong anti-FrCas^E response with both humoral and CTL arms. Similarly, results obtained in the FV model, using transgenic mice (DEREG mice, which could not be used in our study as they were generated in the C57BL/6J genetic background that is resistant to FrCas^E) where Tregs can be selectively ablated, showed that transient Treg depletion permitted exhausted CD8^+ T cells to regain antiviral functions^44. Interestingly, transient depletion of Tregs via administration of an anti-CD25 mAb to FIV-infected cats could also improve the antiviral CTL response but had no effect on the antiviral humoral response^28. Taken with our results, these data indicate that, despite possible species/system differences, the in vivo manipulation of Tregs may be used to strengthen immunotherapeutical approaches aiming at containing viral propagation and counteracting disease progression^20,22,24.

Our most striking observation is that the mAb-based immunotherapy rapidly inhibits the induction of Tregs normally associated with FrCas^E infection. This inhibitory effect is sustained over time and is necessary for the emergence of protective humoral and CTL antiviral immunities, as shown by adoptive transfer of Tregs from infected/non-treated mice that abrogates anti-FrCas^E immunity in infected/667-treated animals. To our knowledge, this is the first time that such a Treg-inhibiting effect is reported for a mAb-based immunotherapy not directly targeting this cell subset, whether this concerns antiviral or anticancer therapy. Several non-exclusive mechanisms may explain the absence of induced Tregs in the presence of the 667 mAb. As Treg expansion in infected/non-treated mice is a consequence of viral
infection via still-to-be-elucidated mechanisms, a first possibility to consider is that limiting the amount of the agent (viral load) causing their induction by a neutralizing mAb may prevent Treg response induction. Supporting this idea, the prevalence of Tregs in lymphoid tissues has been shown to correlate with viral load \(^4\) and disease progression in HIV-infected patients \(^4\). Although not excluding such a contribution, we do not feel that this passive mechanism is sufficient to explain the inhibition of Treg expansion. As such an inhibitory effect is dependent on the effector function of 667, as demonstrated by the presence of an induced Treg response in infected/672-treated- and infected/667-F(ab')\(_2\)-treated mice, an active mechanism(s) involving the formation of immune complexes (ICs) between the neutralizing mAb and its cognate antigen appears more likely. We have previously reported a crucial role for the Fc\(\gamma\)R-binding function of 667 and ruled out a significant contribution for its complement-binding function in the induction of protective antiviral immunity \(^23,24\). Moreover, we have also described a critical role for the interaction between dendritic cells and ICs involving infected cells in this effect \(^24\). It is therefore possible that enhanced activation of DCs by cellular ICs may, not only favor the activation of antiviral CTLs, but also tip the balance of CD4\(^+\) T helper/Treg towards Treg differentiation. Also underlining a possible role for DCs in counteracting the appearance of induced Tregs, Balkow et al. \(^46\) reported that, in the FV model, DC precursors are infected and do not fully mature into DCs, which is crucial for expansion of FoxP3\(^+\) Tregs. Should this also occur in FrCas\(^E\)-infected/non-treated mice, it is likely that limitation of viral spread upon the administration of 667 would also help preserve DC function to generate an efficient antiviral response.

In conclusion, Treg-mediated dysfunction of immune effector cells is a matter of concern in the case of life-threatening chronic viral infections \(^47\) and \textit{in vivo} manipulation of Tregs represents a new therapeutical challenge to strengthen antiviral immunity \(^48,49\). In this context, our herein data suggest that mAb-based immunotherapies directed to viral antigens, owing to their immunomodulatory actions, may constitute invaluable tools to combat the deleterious effect of Tregs in chronic viral infection. As Tregs can anergize anticancer immune responses \(^50\) and as mAb-immunotherapies targeting tumor cell surface molecules may enhance anticancer immune responses \(^25,26\), our findings may also find applications in the treatment of cancer.
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AUTHORSHIP

L. Gros and M. Piechaczyk conceived the study. R. Nasser and L. Gros designed and performed the experiments. M. Pelegrin contributed to data interpretation and corrections of the manuscript. M. Plays performed the follow up of animals and some *in vivo* experimental procedures. M. Piechaczyk and L. Gros wrote the manuscript.

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REFERENCES

9. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. Science. 2008;320:1220-1224


LEGENDS TO FIGURES

Figure 1: Treg responses in immunotherapy-treated and -non-treated infected mice. (A) Flow cytometry analyses of Treg responses at week 8 of age. Mice were infected neonatally with FrCas\textsuperscript{E}. Infected mice were immunotherapy-treated shortly after infection using repeated administration of either 667, 667-F(ab')\textsubscript{2} or 672 for 5 days (see Materials and Methods) and compared to control non-infected/non-treated and infected/non-treated animals. Treg levels were assayed by flow cytometry 8 weeks later on the basis of CD4, CD25\textsuperscript{high} and FoxP3 positivity and were expressed as % of total CD4\textsuperscript{+} T cells. (B) Statistical analysis of Treg responses at week 8 of age. Three experiments, comprising 3 mice each, were conducted as described in A. The data are presented as means ± SEM of Tregs expressed in percent of total CD4\textsuperscript{+} T cells. The statistical significance between groups was established using the Student's t test (**p < 0.01). (C) Treg development in thymus, spleen and lymph nodes of non-infected/non-treated, infected/non-treated and infected/667-treated mice. The different groups of mice were infected and treated as in A. Tregs were assayed as in A and B in total CD4\textsuperscript{+} cells recovered from each organ. Data are presented as means ± SEM of 3 independent experiments conducted with 2 mice per time point. (D) Infection kinetics in thymus, spleen and lymph nodes of infected/non-treated mice. Mice were infected and cells were then prepared, at various time points, from spleen, thymus and lymph nodes for flow cytometry quantification of Env expression at their surface, as described in Materials and Methods. Values are the means ± SEM of 3 independent experiments conducted with 3 mice per condition. (E) Effect of FrCas\textsuperscript{E} viral challenge on Treg levels in infected/non-treated mice. Mice were infected and subjected to viral challenge 8 weeks later, as described in Materials and Methods, and Treg levels were assayed after another 8 days, as in A and B. Values are the means ± SEM of 3 experiments conducted with 3 mice each. The statistical significance between groups was established using the Student’s t test (*p <0.05).

Figure 2: CTL responses in infected/non-treated mice and infected/immunotherapy-treated animals. (A) Expression of IFN\textgamma\textsuperscript{+} in CTLs. Mice were infected neonatally and immunotherapy treated as indicated and IFN\textgamma was assayed by flow cytometry in CD8\textsuperscript{+} T cells in 8 week-old mice. Three mice per group were used in 3 independent experiments. Data are presented as means ± SEM. The statistical significance between groups was established using
the Student’s t test (**p < 0.01). (B) Expression of granzyme B (Gzm) in CTLs. Experiments were conducted as in A with an anti-Gzm antibody. Kinetic expression of PD-1 at the surface of CD8⁺ T cells in spleen (C) and lymph nodes (D). Mice were neonatally infected and 667 immunotherapy-treated, or not, and PD-1 was assayed by flow cytometry at the surface of CD8⁺ T cells at various time points. Values are the means ± SEM of 3 independent experiments conducted with 2 mice per time point. Data corresponding to 667-F(ab')₂- and 672-treated mice are presented in Supplementary Figure 2.

Figure 3: Restoration of the anti-FrCasE immune response in infected/non-treated mice upon Treg depletion. (A) Experimental design. Mice were infected perinatally. At 8 weeks of age, they were re-challenged to facilitate immune response analysis, which was followed by Treg depletion by repeated injections of an anti-CD25 mAb. Anti-FrCasE IgGs and D²-GagL⁺CD8⁺ T cells were assayed 10 days after challenge. To assay the in vivo anti-FrCasE CTL activity, a mix of splenocytes loaded with the GagL peptide or a control peptide was administered 9 days after challenge and death of GagL-loaded cells was assayed on the following day, as described below and in Materials and Methods. (B) Depletion of Tregs. Mice were treated as described in A. CD25 highCD4⁺ T cells were assayed by flow cytometry analysis for FoxP3 positivity at the moment of immune response analysis. (C) Anti-FrCasE immunoglobulin concentrations. Antiviral IgG concentrations were assayed by ELISA in sera of mice 10 days post-rechallenge, as described in A. Three independent experiments with 5 mice per group were conducted. Data are presented as means ± SEM. The statistical significance between groups was established using the Student’s t test (*p < 0.05). (D-E) Flow cytometry assay of D²-GagL tetramer⁺CD8⁺ T cells in Treg-depleted and Treg non-depleted infected/non-treated mice. Analyses were carried out 10 days post-challenge as described in A. (D) corresponds to a representative flow cytometry analysis of CD8⁺ splenocytes of an individual mouse. (E) corresponds to means ± SEM of 3 independent experiments with 5 mice per group. The statistical significance between groups was established using the Student’s t test (*P < 0.05). (F-G) In vivo assay of anti-FrCasE-infected cell CTL responses. FrCasE-infected splenocytes were labeled with the CFSE vital dye at a 10X concentration and splenocytes recovered from naïve mice were labeled with CFSE at a 1X concentration. Both types of splenocytes were mixed in an approximately 1:1 ratio and the mixture was administered to Treg-depleted and Treg-non-depleted infected/non-treated mice on day 9 after challenge. Cell death was monitored on the following day by CFSE flow
cytometry assay of splenocytes recovered from sacrificed mice. F represents an individual mouse analysis. Left peaks correspond to non-infected splenocytes whereas right peaks correspond to infected splenocytes. The decrease of the right peak in the presence of the anti-CD25 mAb is indicative of CTL activity against FrCasE-infected cells. G corresponds to means ± SEM of 3 independent experiments with 5 mice per group. The statistical significance between groups was established using the Student’s t test (**p < 0.01).

**Figure 4: Disruption of anti-FrCasE immune response in infected/667-treated mice upon transfer of Tregs from infected/non-treated mice.** (A) Experimental design. Mice were infected neonatally and immunotherapy-treated with 667. They were virally challenged at 8 weeks of age and subjected to adoptive transfer with purified Tregs (3 x 10^5 cells) from various origins before analysis of humoral and CTL immune responses, conducted as in Figure 3. Tregs were purified from 8 week-old non-infected/non-treated-, infected/non-treated-, infected/667-treated-, infected/667-F(ab’)_2-treated- and infected/672-treated mice. (B) Assay of GagL-specific CD8^+ T cells. (C) In vivo assay of anti-FrCasE-infected cell CTL responses. (D) Assay of anti-FrCasE IgGs. For B, C and D, 3 independent experiments with 3 mice per group were conducted. Data are presented as means ± SEM. The statistical significance between groups was established using the Student’s t test (*p < 0.05).

**Figure 5: Phenotyping of Tregs from infected/non-treated and infected/667-treated mice and the role of IL-10 produced by Tregs from infected/non-treated mice.** (A) Phenotyping of Tregs. Tregs were prepared from 8 week-old non-infected/non-treated, -infected/non-treated- or -infected/667-treated mice and subjected to TCR stimulation, as described in Materials and Methods, before flow cytometry analyses of the indicated proteins. Two independent experiments with 3 mice per group were conducted. Data are presented as means ± SEM. The statistical significance between groups was established using the Student’s t test (*p < 0.05; **p < 0.01). (B-D) Role of IL-10 in Tregs from infected/non-treated mice. Tregs were purified from 8 week-old infected/non-treated mice and grafted to infected/667-treated mice of the same age. These mice were virally challenged at the same time, as described in Materials and Methods. Half of the animals were subjected to intraperitoneal administration of a neutralizing anti-mouse IL-10 mAb from day 5 to day 10 post-viral challenge/Treg grafting whereas the other half was left untreated. Immune response analyses were carried out on day 10 post-grafting as in Figure 4 and the experiments were conducted as described in Materials and Methods. (B) Assay of GagL-specific CD8^+ T cells.
(C) In vivo assay of anti-FrCasE-infected cell CTL responses. (D) Assay of anti-FrCasE IgGs. Two independent experiments with 4 mice per group were conducted. Data are presented as means ± SEM. The statistical significance between groups was established using the Student’s t test (*p < 0.05; **p < 0.01).
Figure 1
Figure 2
Figure 5
Control of regulatory T cells is necessary for vaccine-like effects of antiviral immunotherapy by monoclonal antibodies

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