

B cell depletion therapy dampens CD8+ T cell response in ANCA-associated vasculitis.

Running title : B cell – CD8 T cell axis in ANCA+ vasculitis

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Conflict of Interest : None

Abstract

Objective: To compare the effects of rituximab (RTX) and conventional immunosuppressants (CIS) on CD4⁺, regulatory (Treg) and CD8⁺ T cells in ANCA-associated vasculitis (AAV).

Methods: Thorough immunophenotypic analysis of CD4⁺, Treg and CD8⁺ cells of 51 AAV patients. Cytokine/chemokine production of *in vitro* stimulated CD8⁺ T cells was assessed using a multiplex immunoassay. AAV B cell impact on CD8⁺ T cell response was assessed using autologous and heterologous co-cultures.

Results: CD4⁺ and Treg cell subsets were comparable among RTX and CIS treated patients. By contrast, within the CD8⁺ T cell compartment RTX, but not CIS, reduced CD45RA⁺CCR7⁺ (TEMRA) cell frequency and efficiently dampened cytokine/chemokine production. CD8⁺ T cells co-cultured with autologous B cells produced more pro-inflammatory cytokines in AAV patients than in controls. *In vitro* disruption of AAV B cell – CD8⁺ T cell crosstalk reduced CD8⁺ T cell cytokine production, mirroring the reduced CD8⁺ response observed *ex vivo* after RTX.

Conclusions: The disruption of a pathogenic B cell - CD8⁺ T cell axis may contribute to the efficacy of RTX in AAV. Further studies are needed to determine the value of CD8⁺ T cell immunomonitoring in B cell targeted therapies.

Keywords:

ANCA-associated vasculitis, rituximab, B cells, Cytotoxic CD8⁺ T cells, CD4⁺ T cells

Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitides (AAV) are auto-immune diseases characterized by small vessel and/or extravascular inflammation. Pathogenesis involve both innate and adaptative immunity (1,2) including ANCA-producing B cells, CD4⁺ and regulatory T cells (Treg) (3,4). The analysis of AAV patients leucocyte transcriptome and mouse models suggest that CD8⁺ T cell are also involved (5,6). How this complex auto-immune response is orchestrated remains unclear. In 2010 and 2014 two randomized trials demonstrated the efficacy of rituximab (RTX) in AAV, and thus the central role of B cells (7,8). The initial rationale for targeting B cells was that anti-MPO ANCA are pathogenic (3). However T cells likely contribute to tissue damage (3). B cells can present antigens, produce various cytokines and engage bi-directional crosstalks with other immune cells, including T cells. Their antibody-independent pathogenic role in auto-immunity has been demonstrated years ago (9). In experimental encephalitis, the efficacy of RTX has been linked to the suppression of IL-6 producing B cells (10). In human diseases, most investigators have focused on the impact of RTX on CD4⁺ T cells, with conflicting results (11). How RTX impacts T cell response in AAV patients has not been fully investigated (12).

In 2010-2014 AAV maintenance treatment shifted from conventional immunosuppressant (CIS) to RTX in France. We viewed this transitional period as a unique opportunity to compare the effect of RTX and CIS on T cells and improve our understanding of the remarkable efficacy of RTX.

Material and Methods

Inclusion criteria and blood samples

Inclusion criteria were a diagnosis of ANCA+ MPA or GPA. EGPA patients and those on dialysis were excluded. Patients with active disease received no treatment (including steroids). Remission was defined by a BVAS=0 for 3 months with no evidence of systemic inflammation, that allowed stable or tapered maintenance treatment. Patients in remission received either no treatment, maintenance RTX (500mg every 6 months) or oral CIS (azathioprine, mycophenolate or methotrexate), +/- low dose corticosteroids (<10mg/d) for >3 months. The study was performed in accordance with Helsinki declaration, with participants' written informed consent. PBMCs were separated on a Ficoll gradient layer and frozen in 10% DMSO autologous serum. The presence of anti-CMV IgG was determined by chemiluminescent immunoassay (DiaSorin) on frozen sera.

Polychromatic Flow Cytometry

Detailed phenotype of T cell subsets was performed using 4 mAb panels analyzed with an LSRII flow cytometer (BD Immunocytometry Systems). 1/ CD4⁺ T cell panel: CD3 (OKT3; Brilliant Violet 605), CD4 (L200; PerCP-Cy5.5), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CCR4 (1G1; PE), CCR5 (2D7/CCR5; FITC) and CD161 (191B8; PE-Vio770). 2/ Treg cell panel: CD3 (OKT3; Brilliant Violet 605), CD4 (RPA-T4; Pacific Blue), CD25 (BC96; Brilliant Violet 421), CD127 (A019D5; PE), FoxP3 (236A/E7; Alexa Fluor 647), Helios (22F6; Alexa Fluor 488), CD45RA (T6D11; APC-Vio770) and CD161 (191B8; PE-Vio770). 3/ CD8⁺ T cell panel #1. CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605), CD28 (CD28.2; PE-CF594), CD57 (TB03; FITC) and T-bet (O4-46; PE). 4/ CD8⁺ T cell panel #2: CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605), CD28 (CD28.2; PE-

CF594), GZM-B (GB11; Alexa Fluor 700) and PERF (B-D48; PE). Intracellular cytokine staining was performed using a Fixation / Permeabilization Kit (BD Biosciences) and mAb IL-2 (5344.111; PE), TNF- α (6401.1111; FITC).

Yellow LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.0.1 (TreeStar). All the antibodies were purchased from BD Biosciences, except for anti-CD3, -CD8, -CD45RA, -CD161 and -CD57 mAbs (Miltenyi), anti-Helios, Brilliant violet anti-CD3 and -CD25 (BioLegend), anti-FoxP3 (eBioscience) and anti-PERF mAb (Diacclone).

CCR7 and CD45RA expression were used to identify naïve (CD45RA⁺CCR7⁺; Tn), central memory (CD45RA⁻CCR7⁺; Tcm), effector memory (CD45RA⁻CCR7⁻; Tem) and late differentiated effector memory (CD45RA⁺CCR7⁻; TEMRA) CD4⁺ or CD8⁺ T cells.

FACS sorting, cell culture and multiplex cytokine production measurement

PBMCs were thawed, rested overnight in TexMacs medium (Miltenyi) and stained with anti-CD3, -CD4, -CD8 mAbs. CD3⁺CD4⁻CD8⁺ were FACS-sorted (FACSAria, BD Biosciences; purity greater than 95%) and then stimulated for 4 hours with PMA (100ng/ml; Sigma) and Ionomycin (1 μ g/ml; Sigma) in 96-well round bottom plates (5x10⁵cells/well) at 37°C in 5% CO₂. The production of 34 cytokines and chemokines was measured in CD8⁺ T cell culture supernatant using a multiplex Luminex immunoassay (ProcartaPlex Human Cytokine & Chemokine Panel 1A ; Affymetrix).

B cell – CD8⁺ T cell cocultures

B cells (CD19⁺), naïve (CD45RA⁺CD28⁺) and effector memory (CD45RA⁻CD28⁺) CD8⁺ T cells were purified by FACS (FACSAria, BD Biosciences; purity greater than 95%). B cells were cultured with naïve or effector memory CD8⁺ T cell (1:1 ratio, 10⁶ cell/mL in round bottom plate) in TexMACS medium with Staphylococcal Enterotoxin B (10 mg/ml). After 72h, brefeldin A (10mg/ml, Sigma) was added for 4 hours. IL-2 and TNF- α production of CD8⁺ T cells was then assessed by intracellular flow-cytometry. All AAV patients had active untreated disease (no steroids, no immunosuppressant, no previous RTX, n=15).

Statistical analysis

Quantitative variables are presented as median (interquartile range). Unpaired group comparisons were performed using Mann-Whitney test (2 groups) or Kruskal-Wallis with Dunn's post-test (3 groups or more). Paired data were compared using Wilcoxon signed-rank test.

Results

Cohort characteristics

A total of 51 patients were included. Clinical data are reported in **Table 1**. Among 63 samples, 20 were obtained from patients with untreated active disease (at diagnosis in 18 cases) and 43 from patients in remission under maintenance treatment with either RTX (n=20) or CIS (n=14, AZA in 10, MTX in 2 and MMF in 2), or during drug free remission (n=9). Twelve patients were analyzed at 2 time points, first while untreated (active disease) and then in remission under RTX (n=8) or CIS (n=4). All RTX-treated patients had received a 500mg infusion 6 months before sampling and had undetectable B cells (<5/mm³). Median B cell count of CIS patients was 42/mm³ (IQR:10-56). Median CD4⁺ and CD8⁺ T cell count

was 292 (255-343) versus 344 (211-644) and 199 (160-308) versus 238 (145-324), in CIS versus RTX patients, respectively (non significant).

Most key clinical data were comparable among patients treated with RTX vs CIS, including demographics, disease and ANCA type, past exposure to cyclophosphamide and renal function (**Table 1**). Patients treated with RTX tended to have suffered more relapses as compared to patients receiving CIS (not statistically significant). By contrast, drug free patients had more prolonged remission duration as compared to CIS or RTX treated patients (median: 46 vs 10 and 7 months, respectively, $p=0.01$ and 0.001). Furthermore, most of these 9 Long Term Remission Off Therapy (LTROT) patients had suffered a unique disease flare before entering remission, whereas patients treated with CIS or RTX had a more frequent history of relapse (70%, for RTX, 12% for LTROT, $p=0.01$).

Remission patients on conventional immunosuppressant or rituximab exhibit similar CD4⁺ and Treg cell subsets distributions.

We first assess whether the disease activity and/or the maintenance therapy impact the distribution of the different CD4⁺ and Treg cell subsets. Patients in remission had a decreased proportion of naïve (CD45RA⁺CCR7⁺; T_n) CD4⁺ T cell (**Figure 1A**; $p=0.002$) and an increase in effector memory (CD45RA⁻CCR7⁻; T_{em}) cells (**Figure 1A**; $p=0.001$) as compared to patients with active disease. When the analysis was narrowed to remission patients, we found that RTX, CIS and LTROT patients had comparable distributions of CD4⁺ T cell subsets. The expression of CCR5, CCR4 and CD161 was then monitored as surrogate markers for Th1, Th2 and Th17 cells respectively. As expected, CCR5, CCR4 and CD161 were mainly expressed on effector memory cells (**Figure 1B**). Their level of expression was analyzed on total CD4⁺ cells as well as on T_n, T_{cm}, T_{em} and TEMRA subsets. No difference was observed between CIS, RTX and LTROT patients (**Figure 1C**).

Several groups, including ours, have reported on quantitative and/or functional Treg deficiency in AAV (4) and a recent study suggested that RTX increased Treg frequency in patients with GPA (11). In our cohort, the frequency of CD25^{hi}CD127^{lo}FoxP3⁺ Treg was not significantly different in CIS, RTX and LTROT patients (**Figure 2B**, $p=0.195$). We hypothesized that RTX could target some Treg cell subsets that have been identified in the recent years including resting (CD45RA⁺), memory (CD45RA⁻FoxP3^{hi}) Treg, or Treg subsets that reportedly exhibit a pro-inflammatory potential, i.e. CD161⁺ Treg (14-15) or Treg lacking Helios expression (16-18). We thus analyzed these subsets (gating strategy : **Figure 2A**), but failed to find any difference between RTX and CIS treated patients (**Figure 2C**). Overall, RTX had no distinctive impact on CD4⁺ and Treg cells subset distribution as compared to CIS.

Remission patients on conventional immunosuppressant or rituximab exhibit opposite CD8⁺ T cell subsets distributions.

In contrast to CD4⁺ and Treg cell compartment, we observed significant differences in CD8⁺ T cell subsets distribution across CIS, RTX and LTROT groups (**Figure 3A**). RTX and LTROT patients had less CD45RA⁺CCR7⁻CD8⁺ (TEMRA) and more CD45RA⁻CCR7⁺CD8⁺ (Tem) cells than CIS patients. We then assessed whether the phenotypic features of TEMRA CD8⁺ cells were different across these 3 groups. As expected, TEMRA CD8⁺ T cells were predominantly CD28⁻CD27⁻ with a high expression of perforin, granzyme B and CD57, (**Figure 3B**). No significant differences among treatment groups were seen for the expression of these markers on TEMRA CD8⁺ cells (**Figure 3C**).

Given that the decision to use RTX or CIS was left to the discretion of the clinician, we assessed whether any confounding clinical parameter correlated with TEMRA frequency. Renal function, disease type, ANCA specificity, disease duration and the number of past disease flares did not correlate with TEMRA CD8⁺ T cells frequency (**Figure 3D**). Recent studies have revealed that CD4⁺ and CD8⁺ TEMRA frequency is critically influenced by latent CMV infection in AAV (19,20). Accordingly, we found that latent CMV infection was associated with an increased proportion of TEMRA cells. Among the entire cohort, the frequency of TEMRA CD8⁺ cells was 20.5% and 48.5% in CMV⁻ and CMV⁺ patients, respectively (p<0.001). In order to confirm the opposite effect of RTX and CIS on CD8⁺ memory cells and to rule out any confounding effect of CMV status, we analyzed the CD8⁺ T cell phenotype of 12 patients before and 6 to 12 months after the initiation of RTX or CIS. This analysis confirmed that, regardless of CMV status, RTX reduced TEMRA CD8⁺ T cells frequency, whereas the CIS tended to have the opposite effect (**Figure 3E**). We then investigated whether cytotoxic CD8⁺ T cells were present in vasculitis lesions using diagnostic muscle biopsy from 3 patients with active untreated AAV. CD8⁺ T cells infiltrating inflamed small vessels were observed in all cases and expressed the cytotoxic marker Tia1. Representative data are shown in **Figure 3F**.

B cell depletion dampens ex-vivo CD8⁺ T cell cytokine production

In order to determine whether the opposite effect of RTX and CIS on the CD8⁺ T cells compartment had functional consequences in AAV patients, we compared their impact on *in vitro* cytokine and chemokine production of purified CD8⁺ T cells stimulated with PMA and ionomycin. We found that CD8⁺ T cells from patients in remission under CIS produced similar levels of cytokine/chemokine than those of patients with untreated active disease. Conversely, CD8⁺ T cells from patients receiving RTX produced lower levels of pro-inflammatory cytokine and chemokines compared to the 2 other groups (**Figure 4A**). Differentially expressed cytokines/chemokines included IFN- γ , MIP-1 α /CCL3, MIP-1 β /CCL4,

RANTES/CCL5, IP-10/CXCL10, SDF-1 α /CXCL12a and Eotaxine. A similar trend was observed with TNF- α and GM-CSF. Low amounts of IL4, IL5, IL8, IL10, IL12p70, IL13, IL18, IL21, IL22, IL27, MCP1 and GRO α were also detectable, without any difference across groups. Levels of IL1- α , IL1- β , IL1Ra, IL6, IL7, IL9, IL15, IL17A, IL23, IL31, IFN- α and TNF- β were below the detection threshold. IL2 production was too high to be confidently quantified.

Thus, B cell depletion therapy appears to be more effective than a pleiotropic CIS in suppressing the pro-inflammatory potential of CD8⁺ T cells. Of note, the production of pro-inflammatory cytokines/chemokines did not correlate with TEMRA CD8⁺ T cells frequency, suggesting that RTX had a broad effect on CD8⁺ T cell compartment, beyond its impact of memory CD8⁺ T cell subsets distribution.

AAV B cells promote CD8⁺ T cell activation *in vitro*

Having shown that B cell depletion reduced TEMRA frequency and CD8⁺ T cell cytokine production *ex vivo*, we thought to assess whether active untreated AAV patients B cells could promote CD8⁺ T cell activation *in vitro*. FACS-sorted naïve or effector memory CD8⁺ T cells were cocultured for 72 hours with B cells in the presence of Staphylococcus aureus Enterotoxin B Superantigen (SEB). Cytokine production of CD8⁺ T cells was then assessed by flow-cytometry with intracellular staining. Preliminary experiments performed in healthy volunteers confirmed that in B cell - CD8⁺ T cell cocultures, the addition of SEB induced a B cell-dependant CD8⁺ T cell activation (**Supplementary Figure 1**).

We found that CD8⁺ T cell cocultured with autologous B cells produced more pro-inflammatory cytokines in patients with active untreated AAV than in age-matched healthy volunteers (HV) (**Figure 4B**). Of note, the cytokine production was lower when CD8⁺ T cell subsets from patients were stimulated with B cells from HV or vice-versa (**Figure 4B**).

Collectively, these results demonstrate the crosstalk between B cell and CD8⁺ T cell in AAV patients, leading to CD8⁺ T cell hyperactivation.

Discussion

Our main objective was to compare the impact of RTX and CIS on CD4⁺ T cells, Treg cells and CD8⁺ T cells in AAV.

The first finding of our study was that patients receiving RTX or CIS had similar CD4⁺ and Treg subsets. CD4⁺ helper T cells are believed to play a key role in AAV (3), including Th1, Th2, Th17 and follicular helper T cells (TFH). Whether B depletion differentially affects these populations remains unclear. In auto-immune thrombocytopenia, Stasi et al. reported an increase in Th2 cell frequency in patients that responded to B cell depletion treatment (21). In rheumatoid arthritis (RA), it has been suggested that B cell depletion reduced the Th17 response but not Th1 cells (22). Recently, Verstappen et al. reported that patients with primary Sjogren syndrome exhibited an increased frequency of TFH cell, which decreased upon B cell depletion, whereas Th1 and Th2 population remained stable. A subtle decrease of Th17 cell was also noticed (23). How RTX impact distinct CD4⁺ T cell lineage probably vary according to the underlying disease and remains to be fully investigated (11). However, when we compared the expression of Th1, Th2 and Th17 markers on CD4⁺ T cells from AAV patient in remission we found no significant difference between RTX and CIS.

Several investigators have reported that AAV patients' Treg cells had a decreased suppressive function (4, 24-26), even in remission and in the absence of any immunosuppressant (4). As for B cell depletion therapy effects on Treg cells, contradictory data have been reported in studies performed in various settings such as cryoglobulinemic vasculitis (27), rheumatoid arthritis (28,29), systemic lupus (30,31), autoimmune

thrombocytopenia (17). Importantly, most of the mentioned studies were based on uncontrolled longitudinal studies using simple staining strategies for Treg cell identification. Herein, we report the result of an in-depth phenotypic analysis of Treg cells of AAV patients. Treg expressing CD161+ or lacking Helios have been reported to be more prone to produce pro-inflammatory cytokines (14-18). We found no clear impact of B cell depletion therapy on these subsets as compared to CIS. Recently, Zhao et al. compared the frequency of Treg cells and CD45RA/CD25 expression defined subsets in GPA patients under various conditions (active vs inactive, CIS vs RTX). They found that the frequency of Treg in GPA patients following RTX was similar to that of healthy controls, while GPA patients receiving CIS had a reduced frequency of Treg cells (6). They also found a trend for Treg frequencies to increase following RTX. By contrast, we found no significant difference in Treg cell frequency in patients receiving RTX or CIS. Of note, the increase in Treg cells (CD4⁺CD25⁺CD127⁻ T cells) noticed by Zhao et al. was mainly driven by CD45RA⁻CD25⁺ cells, which are probably not bona fide Treg (13). We also assessed the frequency of resting (CD45RA⁺), memory (CD45RA⁻FoxP3^{hi}) Treg and found no difference between RTX and CIS treated patients. Interestingly, a recent study demonstrated that in tumor bearing mice, RTX could even dampen Treg expansion (33). Overall, further studies are needed to determine whether RTX impact the Treg cell compartment in patients with autoimmune diseases.

Our main finding was that RTX and CIS had clear opposite effects on CD8⁺ T cells. TEMRA cells were expanded by CIS but decreased under RTX. These cells had a typical TEMRA phenotype with a high cytotoxic potential reflected by the strong expression of Perforin and Granzyme B, which may be implicated in tissue damage. In a murine model pauci-immune glomerulonephritis both anti-CD8 and anti-perforin antibody therapies have been shown to reduce glomerular lesions (34,35). In AAV patients, CD8⁺ T cells are present in granulomatous and renal lesions. However, little data were available regarding the role of CD8⁺ T cells in AAV until recently. In 2010, McKinney *et al.* analyzed the prognostic value of

the transcriptional profiling of neutrophils, monocytes, CD8⁺ T cells, CD4⁺ T cells and B cells in AAV (5). Unexpectedly, they found that the CD8⁺ T cell transcriptome identified two subject groups and predicted relapse risk. They identified a CD8⁺ T cell exhaustion signature that predicted a favorable outcome in a wide range of autoimmune disease. These findings suggest that CD8⁺ T cell effector functions have a deleterious role in AAV and other autoimmune diseases.

Interestingly, we found that B cell depletion therapy reduced AAV patients' CD8⁺ T cell cytokine production, whereas CIS did not. In other words, a B cell targeted therapy had more impact on CD8⁺ T cells cytokine production than an immunosuppressant that directly impact T cell biology. This paradoxical finding suggests that B cells play a key role in the CD8⁺ T cell response, which may contribute to the high efficacy of RTX in AAV. However, as viral and/or opportunistic infections are not a usual feature of the absence of B cell in humans (i.e. X-linked agammaglobulinemia), one may cast doubt over the role of B cells in the CD8⁺ response in human. In fact, protective and auto-immune humoral and cellular responses may not be equally sensitive to B cell depletion. For instance, RTX has been shown to reduce auto-reactive but not tetanus –toxoid specific CD4⁺ cells in pemphigus vulgaris (36).

Unfortunately, AAV relapses remain a key issue in the era of B cell depletion therapy. Several teams have shown that highly sensitive B cell immunophenotyping could help estimate the relapse risk following B cell depletion in SLE (37) or RA (38) and more recently AAV (39-41). However, this B cell focused approach poses technical difficulties due to the rarity of B cell following RTX, and do not assess the pleiotropic effects of B cell repopulation. Our results suggest that the analysis of CD8⁺ T cells during and after RTX maintenance treatment may help identify new immunomonitoring readouts.

In conclusion, the disruption of B cell help to a pathogenic CD8⁺ T cell response could contribute to the dramatic efficacy of RTX. Further studies are needed to determine how B cell impact CD8 T cell responses and to assess the value of CD8⁺ T cells immunomonitoring under B cell targeted therapies.

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References

1. Braudeau C, Néel A, Amouriaux K, Martin JC, Rimbert M, Besançon A, et al. Dysregulated Responsiveness of Circulating Dendritic Cells to Toll-Like Receptors in ANCA-Associated Vasculitis. *Front Immunol* 2017;8: 102.
2. Braudeau C, Amouriaux K, Néel A, Herbreteau G, Salabert N, Rimbert M, et al. Persistent deficiency of circulating mucosal-associated invariant T (MAIT) cells in ANCA-associated vasculitis. *J Autoimmun* 2016;70:73-9.
3. Kallenberg CG, Stegeman CA, Abdulahad WH, Heeringa P. Pathogenesis of ANCA-associated vasculitis: new possibilities for intervention. *Am J Kidney Dis* 2013;62:1176-87.
4. Rimbert M, Hamidou M, Braudeau C, Puéchal X, Teixeira L, Caillon H, et al. Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis. *PLoS One* 2011;6:e18734.
5. McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DR, Willcocks LC, et al. A CD8+ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med* 2010;16:586-91.
6. Chang J, Eggenhuizen P, O'Sullivan KM, Alikhan MA, Holdsworth SR, Ooi JD, et al. CD8+ T Cells Effect Glomerular Injury in Experimental Anti-Myeloperoxidase GN. *J Am Soc Nephrol* 2017;28:47-55.
7. Guillevin L, Pagnoux C, Karras A, Khouatra C, Aumaître O, Cohen P, et al; French Vasculitis Study Group. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med* 2014;371:1771-80.
8. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med* 2010;363:221-32.
9. Chan OT, Hannum LG, Haberman AM, Liu Z, Zhao M, Aratani Y, et al. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J Exp Med* 1999;189:1639-48.
10. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* 2012;209:1001-10.
11. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* 2010;10:236-47.

12. Zhao Y, Lutalo PMK, Thomas JE, Sangle S, Choong LM, Tyler JR, et al. Circulating T follicular helper cell and regulatory T cell frequencies are influenced by B cell depletion in patients with granulomatosis with polyangiitis. *Rheumatology* 2014;53:621-30.
13. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009;30(6):899-911.
14. Afzali B, Mitchell PJ, Edozie FC, Povoleri GA, Dowson SE, Demandt L, et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur J Immunol*. 2013;43(8):2043-54.
15. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood*. 2013;121:2647-58.
16. Verhagen J, Wraith DC. Comment on "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells." *J Immunol* 2010;185:7129.
17. Takatori H, Kawashima H, Matsuki A, Meguro K, Tanaka S, Iwamoto T, et al. Helios Enhances Treg Cell Function in Cooperation With FoxP3. *Arthritis Rheumatol* 2015;67:1491-502.
18. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010;184:3433-41.
19. Morgan MD, Pachnio A, Begum J, Roberts D, Rasmussen N, Neil DA, et al. CD4+CD28- T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality. *Arthritis Rheum* 2011;63:2127-37.
20. Eriksson P, Sandell C, Backteman K, Ernerudh J. Expansions of CD4+CD28- and CD8+CD28- T cells in granulomatosis with polyangiitis and microscopic polyangiitis are associated with cytomegalovirus infection but not with disease activity. *J Rheumatol*. 2012;39:1840-3.
21. Stasi R, Cooper N, Del Poeta G, Stipa E, Laura Evangelista M, Abruzzese E, et al. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood* 2008;112:1147-50.

22. van de Veerdonk FL, Lauwerys B, Marijnissen RJ, Timmermans K, Di Padova F, Koenders MI, et al. The anti-CD20 antibody rituximab reduces the Th17 cell response. *Arthritis Rheum*. 2011;63:1507-16.
23. Verstappen GM, Kroese FG, Meiners PM, Corneth OB, Huitema MG, Haacke EA, et al. B Cell Depletion Therapy Normalizes Circulating Follicular Th Cells in Primary Sjögren Syndrome. *J Rheumatol*. 2017;44:49-58.
24. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CGM. Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. *Arthritis Rheum* 2007;56:2080-91.
25. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology*. 2010;130(1):64-73.
26. Free ME, Bunch DO, McGregor JA, Jones BE, Berg EA, Hogan SL, et al. Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum*. 2013;65(7):1922-33.
27. Saadoun D, Rosenzweig M, Landau D, Piette JC, Klatzmann D, Cacoub P. Restoration of peripheral immune homeostasis after rituximab in mixed cryoglobulinemia vasculitis. *Blood* 2008;111:5334-41.
28. Feuchtenberger M, Müller S, Roll P, Waschbisch A, Schäfer A, Kneitz C, et al. Frequency of regulatory T cells is not affected by transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Open Rheumatol J*. 2008;2:81-8.
29. Díaz-Torné C, Ortiz de Juana MA, Geli C, Cantó E, Laiz A, Corominas H, et al. Rituximab-induced interleukin-15 reduction associated with clinical improvement in rheumatoid arthritis. *Immunology* 2014;142:354-62.
30. Vigna-Perez M, Hernández-Castro B, Paredes-Saharopulos O, Portales-Pérez D, Baranda L, Abud-Mendoza C, et al. Clinical and immunological effects of Rituximab in patients with lupus nephritis refractory to conventional therapy: a pilot study. *Arthritis Res Ther* 2006;8:R83.
31. Vallerskog T, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, et al. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol* 2007;122:62-74.

32. Stasi R, Cooper N, Del Poeta G, Stipa E, Laura Evangelista M, Abruzzese E, et al. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. *Blood* 2008;112:1147-50.
33. Deligne C, Metidji A, Fridman W-H, Teillaud J-L. Anti-CD20 therapy induces a memory Th1 response through the IFN- γ /IL-12 axis and prevents protumor regulatory T-cell expansion in mice. *Leukemia* 2015;29:947-57.
34. Fujinaka H, Yamamoto T, Feng L, Nameta M, Garcia G, Chen S, et al. Anti-perforin antibody treatment ameliorates experimental crescentic glomerulonephritis in WKY rats. *Kidney Int* 2007;72:823-30.
35. Reynolds J, Norgan VA, Bhambra U, Smith J, Cook HT, Pusey CD. Anti-CD8 monoclonal antibody therapy is effective in the prevention and treatment of experimental autoimmune glomerulonephritis. *J Am Soc Nephrol* 2002;13:359-69.
36. Eming R, Nagel A, Wolff-Franke S, Podstawa E, Debus D, Hertl M. Rituximab exerts a dual effect in pemphigus vulgaris. *J Invest Dermatol* 2008;128:2850-8.
37. Vital EM, Dass S, Buch MH, Henshaw K, Pease CT, Martin MF, et al. B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis Rheum* 2011;63:3038-47.
38. Dass S, Rawstron AC, Vital EM, Henshaw K, McGonagle D, Emery P. Highly sensitive B cell analysis predicts response to rituximab therapy in rheumatoid arthritis. *Arthritis Rheum* 2008;58:2993-9.
39. Md Yusof MY, Vital EM, Das S, Dass S, Arumugakani G, Savic S, et al. Repeat cycles of rituximab on clinical relapse in ANCA-associated vasculitis: identifying B cell biomarkers for relapse to guide retreatment decisions. *Ann Rheum Dis*. 2015;74:1734-8.
40. Bunch DO, McGregor JG, Khandoobhai NB, Aybar LT, Burkart ME, Hu Y, et al. Decreased CD5⁺ B cells in active ANCA vasculitis and relapse after rituximab. *Clin J Am Soc Nephrol* 2013;8:382-91.
41. Bunch DO, Mendoza CE, Aybar LT, Kotzen ES, Colby KR, Hu Y, et al. Gleaning relapse risk from B cell phenotype: decreased CD5⁺ B cells portend a shorter time to relapse after B cell depletion in patients with ANCA-associated vasculitis. *Ann Rheum Dis* 2015;74:1784-6.

Table 1 : Patients characteristics

	<i>Active disease</i>		<i>Remission</i>	
	Untreated (ACT, N=20)	B Cell Depletion Therapy (RTX, N=20)	Conventional Immunosuppressant (CIS, N=14)	Remission Off Therapy (LTROT, N=9)
Male	13 (65%)	14 (70%)	5 (36%)	2 (22%)
Age	67 (55-79)	65 (56-75)	68 (59-82)	63 (62-73)
GPA	11 (55%)	14 (70%)	7 (50%)	6 (66%)
Anti-MPO	11 (55%)	7 (35%)	7 (50%)	4 (44%)
eGFR (ml/min/1,73m²)	53 (17-92)	58 (34-86)	67 (44-93)	59 (34-82)
Disease duration (months)	0.6 (0.3-0.9)	20 (10-108)	19 (13-53)	58 (13-86)
Past CYC exposure	2 (10%)	17 (85%)	11 (79%)	7 (78%)
Remission duration (months)	NA	6 (5-8)	10 (6-17)	46 (20-73)*
Single flare	18 (90%)	7 (35%)	8 (57%)	8 (88%) [†]
Previous flares (n)	0 (0-0)	2 (1-4.5) [‡]	1 (1-2)	1 (1-1)

Figures indicate ratios (percentage) or median (interquartile range). eGFR : estimated glomerular filtration rate;

CYC: cyclophosphamide ; LTROT: Long Term Remission Off Therapy.

*p=0.002 vs RTX; [†] p=0.01 vs RTX; [‡] p=0.008 vs LTROT.

Figure Legends

Figure 1: During remission, maintenance treatment type has no significant impact on CD4⁺ T cell subsets or CD4⁺ helper T cell markers.

(A) Frequency of 4 CD4 T cell subsets according to the expression of CD45RA and CCR7 in AAV patients. T cells subsets frequencies were analyzed according to disease activity and maintenance treatment. CD4 T cell subsets frequency correlated with disease activity status (** $p < 0.01$, Mann-Whitney test) but not maintenance treatment type during remission. **(B)** The CD4 T cells surface expression of CCR5, CCR4 and CD161 were used as surrogates for Th1, Th2 and Th17 response, respectively. The expression of these markers was compared between Tn and Tem and across the 3 groups of patients in remission. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Histograms within dot plots represent the median. Boxplot were done using the Tukey method.

Figure 2: During remission, maintenance treatment type has no significant impact on total Treg nor on Treg subsets (A) Gating strategy for the identification of Treg

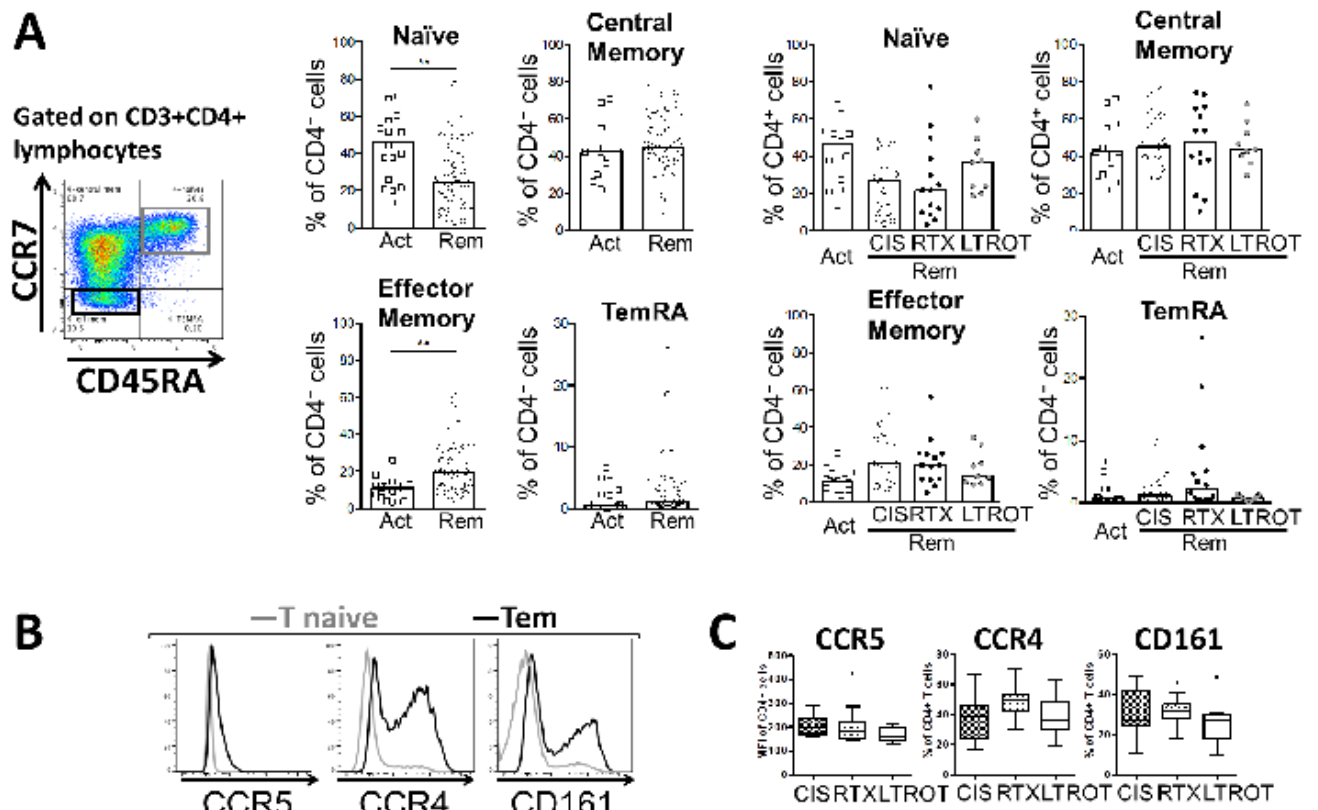
(CD3⁺CD4⁺CD25^{hi}CD127^{lo}FoxP3⁺) and Treg subsets. Resting and memory Treg cells were defined as CD45RA⁺ and CD45RA⁻FoxP3^{hi} Treg, respectively. **(B)** The frequency of Treg and CD161⁺, helios⁺, resting, memory Treg and CD45RA⁻FoxP3^{lo} cells were not significantly different among CIS, RTX and LTROT patients (All $p > 0.05$, Kruskal-Wallis). CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Histograms within dot plots represent the median.

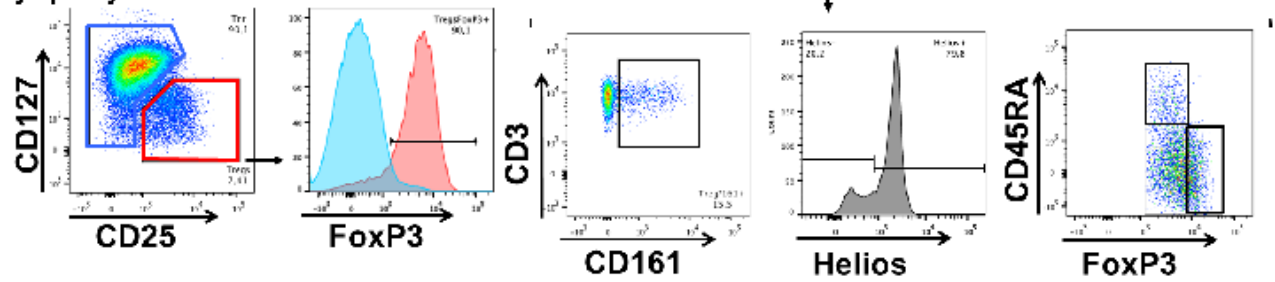
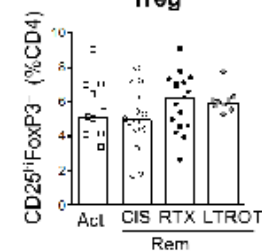
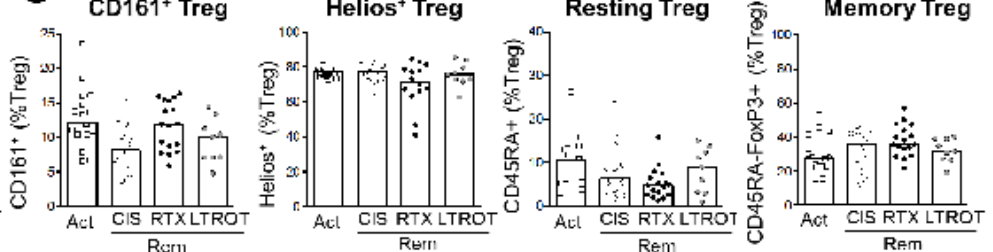
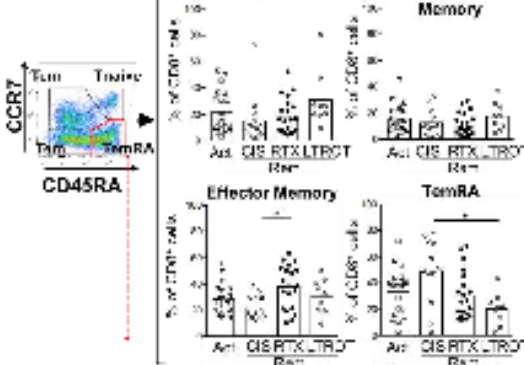
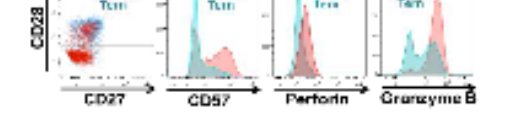
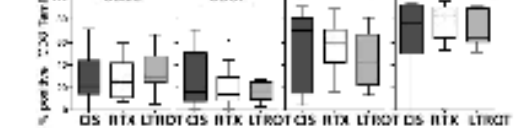
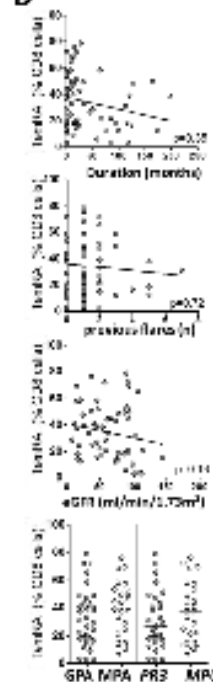
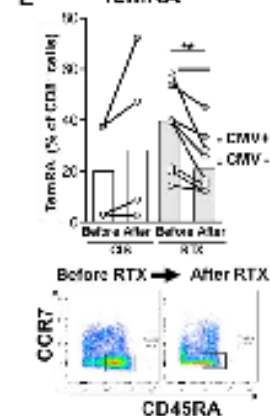
Figure 3. Rituximab and Conventional immunosuppressant have opposite effects on AAV patients' memory CD8 T cells. (A) Frequency of 4 CD8 subsets in subgroups of AAV patients (Act, CIS, RTX and LTROT) according to the expression of CCR7 and CD45RA. Representative flow data is shown. Histogram bar within dot plots represents the median. * $p < 0.05$, Kruskal-Wallis (across remission groups), with Dunn's post-test. **(B)** Representative flow data of phenotypic characterization of TEMRA and Tem. **(C)** Absence of modification of TEMRA phenotype by the maintenance treatment of AAV patients. Bars indicated median \pm SD (CIS, $n=13$; RTX, $n=15$; LTROT, $n=9$). **(D)** Absence of correlation between TEMRA frequency and AAV history, renal function or disease phenotype. **(E)** Longitudinal analysis of TEMRA frequency in AAV patients before and after treatment with RTX ($n=8$) or CIS ($n=4$) and according to the CMV status of patients (open circle, CMV+; gray circle, CMV-). ** $p < 0.01$, Paired **(F)** Immunohistochemical CD8 and Tia1 staining performed on diagnostic muscle biopsy from 3 patients with active untreated AAV. Act : Untreated active disease CIS: conventional immunosuppressant, GPA : granulomatosis with polyangiitis, LTROT: Long term remission off therapy. MPA : microscopic polyangiitis, Rem : remission, RTX: Rituximab.

Figure 4: Diminished *ex vivo* CD8 T cell cytokine/chemokine production of RTX treated AAV patients and propensity of AAV patients B cells to promote CD8 T cell cytokine production *in vitro*.

(A) Decrease of cytokine/chemokine levels secreted by CD8 T cells purified from AAV patients treated with RTX. Cytokine/Chemokine were quantified in the supernatant of purified CD8 T cells after 4h of polyclonal stimulation using a 34plex immunoassay. CD8 T cells from RTX treated patients produced lower levels of pro-inflammatory cytokines/chemokines than CIS treated patients and/or those with active untreated disease (* $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis with Dunn's post-test [Active vs CIS vs RTX]). **(B)** Cytokine production of CD8 T cell purified from active untreated AAV patients or aged-matched HV after 72h of culture

between purified Naïve or EM CD8 T cells and B cells in the presence of *Staphylococcus aureus* Enterotoxin B Superantigen. Autologous cocultures were performed in 29 HV and 11 AAV patients and HV crossed coculture in 15 AAV. CIS: conventional immunosuppressant, RTX: Rituximab, LTROT: Long term remission off therapy. Horizontal bars represent medians.



AGated on CD3⁺CD4⁺ lymphocytes**B****C****A****B****C****D****E****F**