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Running title: Treg and rituximab in membranous nephropathy

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Abstract

Primary membranous nephropathy (PMN) is characterized by antibodies to the podocyte but little is known about B- and T-cell populations and their response to rituximab is controversial. We compared 33 lymphocyte subpopulations and 27 cytokines/chemokines in 25 patients with severe PMN and 27 age-matched healthy controls. At baseline, patients had (i) increased percentage of naive B-cells (p=0.03) with decreased switched (p=0.03) and non-switched (p=0.005) memory B-cells; (ii) decreased percentage of NK cells (p=0.04), with an increase of the CD56^{bright}CD16$^{-/lo}$ NK subset (p=0.002); (iii) decreased percentage of regulatory T cells (p<0.0001), together with an increased plasma concentration of TNF-alpha, IL-5 and IL-2RA. We then investigated patients at 8 days, 3 and 6 months after treatment with rituximab added to supportive therapy (n=16) or supportive therapy alone (n=9) compared to baseline. After rituximab, B-cell recovery was still incomplete at 6 months, with persistent alterations of B-cell subsets, increase of Treg (p<0.05) and NK cell (p=0.05), and decrease of CD56^{bright}CD16$^{-/lo}$ NK subset (p<0.05) and TNF-alpha levels (p<0.01). The patients who clinically responded to rituximab had a lower percentage of Treg at baseline (p=0.01 vs non responders) and an increased percentage at day 8 (p=0.01). Treg remained unchanged in non responders and in patients treated with supportive therapy alone. Evaluation of Treg might be useful for predicting early response to rituximab.

Key words: membranous nephropathy, memory B-cells, regulatory T-cells, NK cells, TNF-alpha, rituximab

INTRODUCTION

Membranous nephropathy (MN) is a major cause of nephrotic syndrome in the adult. It is characterized by the accumulation of immune deposits leading to complement activation on the outer aspect of the glomerular capillary wall. About a third of the patients will undergo spontaneous remission whereas another third will reach end-stage renal failure, and the remaining third will keep various degrees of proteinuria and develop progressive renal failure.

Considerable advances have occurred in the understanding of the pathophysiology of primary MN (PMN) with the identification of three major antigens including neutral endopeptidase in the neonatal disease[1], and the phospholipase A2 receptor (PLA2R)[2] and thrombospondin domain 7A (THSD7A)[3], in the adult disease. All three antigens are expressed on the podocyte where they serve as targets for circulating antibodies. Anti-PLA2R and anti-THSD7A antibodies are detected in about 70 to 80 % and <5 % of adult patients with PMN, respectively[4]. Anti-PLA2R antibodies have been paradigm shifting in diagnosis and patients 'care because of their specificity for MN and their correlation with disease activity[4]. Although correlations have been found between anti-PLA2R antibody titers and outcome, we still cannot explain why patients will undergo spontaneous remission while others will not respond to immunosuppressive agents and develop a severe form of the disease.

At variance with antibodies, circulating lymphocyte populations have been poorly investigated in PMN. Old studies have shown an increase of the $CD4^+$ /$CD8^+$ subset ratio in some patients with MN, with or without nephrotic syndrome[5–12]. In the subset of patients with MN and nephrotic syndrome, some patients had a marked increase in the $CD4^+$ /$CD8^+$ ratio due to a reduction in the CD8 cell subset compared to healthy subjects while others showed a ratio in the normal range[6] It was suggested that a high $CD4^+$/$CD8^+$ ratio might predict clinical response to immunosuppressive treatment[9] although this was not confirmed by later studies in patients treated with rituximab[13]. The imbalance between the $CD4^+$ and $CD8^+$ subsets was associated with decreased suppressor T-cell function, which seems secondary to the nephrotic state[14], and with predominance of the Th2 immune response. Implication of Th2 in pathogenesis of PMN is attested by predominance of IgG4 subclass among circulating and deposited anti-PLA2R and anti-THDSD7A antibodies, increased number of interleukin (IL)-10 or IL-4- secreting T-cells, and increased production of IgG4 by B-cells upon stimulation with Th2 cytokines[2,3,11,15,16].

Surprisingly, there are relatively few data on regulatory T-cells (Treg) in PMN although those cells are known to play a key role in auto-immune diseases[17–19], at least in part by inhibiting

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the activation of helper T-cells induced by antigen-presenting cells, and consequently B-cell activation and production of autoantibodies. Patients with a mutation in FOXP3, a transcription factor which programs the development and function of CD4^{+}CD25^{+} regulatory T-cells, occasionally develop MN[20,21]. Compared with healthy controls, the number of peripheral blood CD4^{+}CD25^{+}Foxp3^{+} regulatory T-cells was reduced in patients with PMN, while the amount of peripheral blood B-lymphocytes was higher, although no correlation was found with proteinuria[12,22]. At variance with these data, Fervenza et al, found that none of the Treg subsets showed quantitative differences, the only persistent change in lymphocyte subsets being a relative increase in natural killer cells[13].

We performed a detailed analysis of B- and T-cell populations in PMN for several reasons. First, we wanted to take advantage of the progress achieved in the characterization of lymphocyte subpopulations in an attempt to solve out the discrepancies previously observed[12,13]. Second, our randomized controlled trial of rituximab plus supportive therapy versus supportive therapy alone made it possible to investigate whether dynamic changes in specific lymphocyte populations might predict response to rituximab in patients with severe PMN[23]. Third, we hypothesized that this study could provide a rational for using novel therapies aimed at increasing Treg such as low-dose IL-2[24].

RESULTS

Patients

The study enrolled 25 patients including 5 females and 20 males with a median age of 57 years (26-74). The male preponderance in the PMN population reflects the higher frequency of PMN in this gender (Table 1). Sixteen patients were enrolled in the NIAT-rituximab group, 9 in the NIAT only group. The imbalance between numbers in each group is related to the randomization process that regulated treatment assignment at the level of the country, which resulted in more patients being enrolled in the NIAT-rituximab group in the Paris area. No difference at baseline was observed between groups regarding age, gender, serum creatinine, serum albumin, proteinuria, and PLA2R-Ab titer (Table 1 and Supplementary Table 1). Among the 16 patients of the NIAT-rituximab group, 9 achieved the primary end point (partial or complete remission) at 6 months, 2 patients of the NIAT group entered spontaneous remission (Table 2).

Anti-PLA2R and anti-THSD7A antibodies at baseline

PLA2R-Abs were detected in 18 of the 25 (72%) patients, including 10 in the NIAT-rituximab group and 8 in the NIAT group (Table 2). Among the 7 PLA2R-Ab-negative patients, 3 had PLA2R-Ag detected in immune deposits; hence the rate of PLA2R-related MN was 84%. Among the 4 remaining patients, one had anti-THSD7A antibody measured by immunofluorescence assay while 3 patients most likely had PMN related to other, as yet unknown antigen(s).

B-, T-, and NK-cell subsets at baseline

While absolute and relative numbers of B cells were comparable in PMN patients and healthy donors, analysis of the B cell subsets shown by CD27 and IgD expression revealed that patients had lower percentages of switched (IgD^-CD27^+) and non-switched (IgD^+CD27^+) memory B cells (20 ± 2% vs 27 ± 2% p=0.03 and 10 ± 1% vs 16 ± 2% p=0.005 respectively) associated with a higher percentage of naive (IgD^+CD27^-) B cells (65 ± 3% vs 54 ± 4% p=0.03). Double negative (IgD^-CD27^-)[25, 26] B cells were also expanded in patients (5.3±0.6 vs 3.6±0.3, p=0.009) (Figure 1A). The percentage of plasmablasts and CD21$^-$ B cells was unchanged compared to healthy donors (Supplementary Table 2).

Among T cell subsets, a decreased frequency of Treg among CD4$^+$ T cells was observed in PMN patients compared to healthy donors (3.2 ± 0.2% vs 4.7 ± 0.2 p<0.0001), (Figure 1B and supplementary Table 2) while Treg absolute numbers were comparable in both groups (Figure

1B). Markers associated with Treg differentiation, activation or function such as CD45RA, Helios, HLA-DR, CTLA-4, GITR or LAP expression were comparable in patients and healthy donors (Supplementary Figure 1 and supplementary Table 2). No correlation was found between Treg% or absolute number, and proteinuria, serum albumin or anti-PLA2R levels at baseline (data not shown). Effector memory CD4$^+$ and CD8$^+$ T cells were increased in patients compared to healthy donors (10 ± 1% vs 6 ± 1% p= 0.03 for CD4+T cells and 26 ± 4% vs 18 ± 1% p= 0.02 for CD8+ T cells), (Supplementary Table 2).

A decreased percentage of NK cells was detected in PMN patients compared to healthy donors (8 ± 1% vs 12 ±1% p=0.04) associated with an increased percentage of CD56^{bright}CD16$^{-/lo}$ NK cells (10 ± 1% vs 6 ± 1% p=0.002), (Figure 1C and supplementary Table 2).

Immunological and clinical outcome in patients treated with NIAT-rituximab

B-cell depletion was achieved at day 8 in all treated patients (2.2 ±0.7 CD19$^+$ cells/mm3 vs239 ±34 at day 0, p<0.0005), persisted at day 90 in all patients except one (14±7 CD19$^+$ cells/mm3, p<0.0001), and CD19 count did not return to baseline level by 6 months (67±20 CD19$^+$ cells/mm3, p<0.001), (Figure 2A). These changes were mostly associated with lack of reconstitution of non-switched memory B-cells that remained very low at day 180 (5±1 % at day 180 vs12±2 % among CD19+ cells at baseline, p=0.008), (Figure 2B).

In the NIAT-rituximab group, 8 of the 9 patients who reached the primary end point (clinical remission) at 6 months had no detectable PLA2R-Ab at 3 months: among them, 3 patients were initially PLA2R-Ab positive and achieved antibody depletion, one patient had a 28-fold lower level of PLA2R-Ab, 4 patients had PLA2R-related MN without detectable antibody at baseline and therefore had probably entered immunological remission, one patient had THSD7A-related MN (Table 2). Conversely, 3 patients without PLA2R-Ab at month 3 had not achieved clinical remission at month 6, which is in accordance with previous observations showing that immunological remission may precede by several months clinical remission[2,27]. In the NIAT group, spontaneous remission occurred in 2 patients with low anti-PLA2R-Ab levels at baseline and absence of detectable antibody at 6 months.

Treg percentage among CD4$^+$ cells and Treg absolute numbers were weakly increased as early as day 8 (reaching statistical significance for Treg percentage), and the average increase reached 22±10% and 36±15%, respectively, at 6 months from baseline (Figure 3A).

A progressive increase of NK-cell percentage was observed (from $8\pm1\%$ at baseline up to $12\pm2\%$ at day 180, p=0.04), (Figure 4A) contrasting with a decrease of

CD56^{bright}CD16$^{-/lo}$ NK cell percentage (from $12\pm2\%$ at baseline down to $7\pm1\%$ at day 180, p=0.03), (Figure 4B). No change was observed in patients treated with NIAT only (Figures 4C and 4D).

Treg cell dynamics might predict early clinical response

Because Treg response to rituximab was heterogeneous, we investigated whether it could be correlated with a different clinical response at 6 months. Responder patients were characterized by a lower percentage of Treg at baseline (responders, 2.4 ± 0.2 vs non-responders, 3.9 ± 0.4, p=0.01), (Figure 3C) whereas all other cell subsets or markers explored in this study were at similar level across patient groups. A significant increase of Treg percentage among CD4$^+$ cells was observed at day 8 (3.01 ± 0.19 vs baseline, 2.57 ± 0.17, p= 0.0015), day 90 (2.78 ± 0.26, p=0.0078) and day 180 (3.46 ± 0.38, p=0.046) in responder patients treated with rituximab, whereas Treg remained unchanged in non-responder patients as well as in patients who were treated with NIAT alone (Figure 3B). Changes in Treg absolute numbers in responder patients showed parallel evolution although significance was reached at month 6 only. There was a slight but significant increase of GITR at month 6 while the other Treg markers were not affected (not shown).

We analyzed correlations between changes in Treg % or absolute numbers, and changes in proteinuria, serum albumin, anti-PLA2R-Ab, NK and CD19 levels at month 6. We found that B (CD19+) cell depletion was correlated (spearman test, p=0.04) with an increase in Treg absolute numbers in responder patients whereas no correlation was observed in non-responders (Figure 5). There was no correlation between Treg levels and absence of PLA2R-Ab or antibody depletion at month 3.

Cytokine profile in PMN patients and outcome with NIAT-rituximab

A panel of 27 cytokines and chemokines was assayed to investigate the Th1/Th2/Th17 balance in PMN patients and healthy donors. A significant increase of IL-5, TNF-α and IL-2RA associated with a decrease of IL-17, IL-1α, IL-7, and GM-CSF was observed in patients compared to healthy controls (Figure 6A and supplementary figures 2 and 3). Among these cytokines, we observed a significant decrease of TNF-α level under treatment from month 3 (Figure 6B), but no difference was observed between responder and non-responder patients

(not shown). The other cytokines including IL-35 were unchanged or could not be assessed at all time points.

DISCUSSION

Studies on B- and T-cell populations in patients with PMN are scarce and their results often controversial. A decrease of Treg was reported in 2 studies from China[12] and Italy[22] whereas a study from the Mayo Clinic found that none of the Treg subsets showed quantitative differences[13]. Roccatello et al [22] showed that in patients treated with rituximab, there was an increase in Treg at month 12, but there was no information on early time points before month 6. The present study features 4 important novelties. First, this is the first such study using detailed CD marker phenotyping of T- and B-cell subsets in the era of autoantibody classification (anti-PLA2R, anti-THSD7A) of PMN cases. Second, it presents a full profile of 26 cytokines in patients with severe PMN at baseline and after rituximab treatment, the major change being an increased level of TNF-alpha followed by a decrease of this cytokine under B-cell depletion. Third, this is the first study performed in a randomized controlled trial which allowed comparison of lymphocyte population outcome in patients treated with rituximab or with supportive therapy. Fourth, it first provides evidence that an early increase in Treg % and absolute values within the first 6 months after rituximab is observed in responder patients only and consequently may be an early predictor of response to rituximab.

Total B-cell numbers and percentages in PMN patients were similar to those of healthy controls. However, the frequency of naive cells was increased while that of switched and non-switched memory cells was decreased. These data are consistent with previous reports in other autoimmune diseases like SLE and Sjogren's syndrome[28, 29]. They indicate that the B-cell compartment undergoes alterations that are apparent in the distribution of the B-cell subsets present in the circulation, which suggests that B-cell tolerance checkpoints are impaired in these autoimmune diseases[29].The lower rate of circulating memory B cells could be explained by over-expression of chemokines that guide them into tissues as suggested by immunochemistry studies showing B-cell infiltration in kidneys[12,29-31], and/or by an increased differentiation of self-reactive B cells into autoantibody producing plasmablasts under the control of T follicular helper (Tfh) cells[29,32]. Exploration of Tfh cells in PMN would be important in future studies.

We observed an increased level of TNF-α in PMN patients followed by a decrease of this cytokine under B-cell depletion. The role of TNF-α in the pathogenesis of PMN is supported by evidence of high plasma and urinary TNF-α levels in patients with PMN[33–35], which was shown to be associated with a polymorphism in the *TNF-α* gene in PMN patients[36]. An increased release of TNF-α by monocytes from patients with PMN has been suggested by

Matsumoto et al[37] but activated B-cells could also contribute by a greater propensity to produce pro-inflammatory cytokines, particularly TNF-α. Given that the B cells reemerging after B-cell depletion are dominated by naive B cells[38,39], it is tempting to speculate that the cytokine normalization is related to B cell depletion as it was suggested in rheumatoid arthritis[26].

We also found changes in the NK subsets, with decreased percentage of NK cells associated with an increase frequency of CD56^{bright}CD16^{-/lo} NK, the later subset being considered as more immature and less cytotoxic than CD56^{+}CD16^{+} NK cells. A reduction in the number of circulating NK cells has been shown in several autoimmune disease like multiple sclerosis, SLE, psoriasis and Graves' disease [40,41].This quantitative defect, usually paralleled by a decrease in NK cell cytotoxicity, gave rise to the hypothesis that circulating NK cells are involved in the control of the autoimmune reaction, as has been shown for regulatory T cells[41].

Treg cells play a fundamental role in the development and maintenance of immunological tolerance, and in most autoimmune diseases, there is an imbalance between harmful self-specific effector T cells (Teffs) that attack normal tissues, and Tregs that normally control them[42,43].Our data show a significant decrease in Treg percentages in PMN patients compared to healthy controls. This is in agreement with previous studies [12,22] and in disagreement with another one[13] in which, however, Treg cells were not analyzed by the expression of the Foxp3 "gold standard marker". Expression of molecules associated with Treg function,i.e GITR, CTLA-4, HLA-DR or LAP, was not affected and we did not observe an imbalance between resting and activated Tregs among total Tregs.

One of the most striking data provided by this study was the kinetics and possible predictive value of Treg cells in patients treated with rituximab. Treg percentage among CD4^{+} T-cells was significantly lower in PMN patients responding to rituximab, and strikingly increased as early as 8 days whereas Treg percentage did not change in the non responders, in whom it remained within the normal range (no significant difference with healthy donors).This is remarkably similar to what we previously observed during the treatment with rituximab of patients with mixed cryoglobulinemia vasculitis[44]. Rituximab was also shown to induce Treg in several other autoimmune disorders such primary thrombocytopenic purpura, lupus nephritis, and rheumatoid arthritis[45-47] as well as in allogenic stem cell transplantation[48]. and this appeared to highly correlate with clinical responses[44,47].These observations are important because they suggest that part of the effects of rituximab in auto-immune diseases and

allogenic settings are linked to an increase in Treg. The precise mechanism for this phenomenon is unclear but might be a consequence of B-cell deprivation as suggested by our own results showing that B (CD19^{+}) depletion was correlated with an increase in Treg absolute numbers in responder patients only. B cells provide signals to T cells through antigen presentation and cytokine production. At least 2 cytokines could be involved. It has been shown that B cells expressing interferon-gamma suppress Treg-cell differentiation and promote autoimmune experimental nephritis, and that interferon-gamma specifically derived from B cells contributes to the pathogenesis of autoimmunity[49]. Another potential candidate is interleukin-35 since IL-35 producing B cells are key players in the negative regulation of immunity[50]. Roccatello et al[22] indeed showed that IL-35 levels were increased compared to baseline values at 6 months after rituximab and that IL-35 did not increase in the patients who did not respond to rituximab. However, we could not confirm these data in the present study. We could not find a difference between patients and healthy donors at baseline, among patients during follow-up, and between responders and non-responders. An alternative mechanism could be the direct depletion of CD20^{dim}Tcells, contributing to decreased T-cell activation[51] or decreased effector T-cell generation which, as a consequence would result in rising Treg percentages among CD4 T cells.

This manuscript puts the focus on B-cells, the main target of rituximab, and Treg cells for their implication in autoimmunity. Further studies should investigate at least 2 important aspects of B- and T-cell pathobiology in patients with PMN, respectively. The first one relates to PLA2R-specific B-cells, particularly memory B-cells. It is well established that switched memory B-cells play a key role in auto-immunity. Upon encounter with the target antigen, B cells differentiate into short- or long-lived, antibody secreting CD20-negative plasma cells and into CD20-positive memory B cells which can differentiate into antibody-secreting cells upon re-exposure to the priming antigen. Since the cell subset responsible for autoantibody production in PMN has not been clearly defined, a role for PLA2R-specific memory B-cells must be considered and their outcome under rituximab treatment may provide important information on these cells as a biomarker of disease activity and a predictor of relapse. A second important issue relates to T cells, including i) identification and functional studies of PLA2R-specific, effector and regulatory T-cells and ii) analysis of the T-cell repertoire. Recent studies on allelotypes associated with MN provide some clues to the nature of PLA2R-specific T-cell epitopes[52] which make the basis for future functional studies.

This study has several limitations. First, it enrolled a relatively small number of patients due to constraints in the recruitment of patients who had to be sampled in the area of Paris to be analyzed in a short time period after sampling. Thus they represent only a third of the patients enrolled in the GEMRITUX trial. Second, the follow-up was limited to 6 months, before occurrence of full B cell reconstitution, because we wanted to give the referring physicians a chance to switch treatment in patients who had not achieved clinical remission at this timepoint, particularly in those from the NIAT group. However, we think that our study lays the ground for further studies which should enroll a greater number of patients over a period of 12 months, and include additional cell populations such as Tfh cells and relevant cytokines/chemokines.

Altogether, our results suggest that an increase of Treg percentage might be a marker of early response to rituximab, possibly prior to PLA2R-Ab decrease. They require confirmation in a prospective study. They should lead to investigate whether an increase of Treg cells is associated with spontaneous remissions of PMN. Finally, they raise the possibility that immuno-intervention aimed at increasing Treg count and functions such as low-dose IL-2[24] could also be beneficial in PMN.

METHODS

Study population

This study presents the results of an ancillary study to the GEMRITUX (Evaluate Rituximab Treatment for Primary Membranous Nephropathy) randomized controlled trial (NCT01508468)[23]. Eligibility criteria, study treatments and monitoring, and definition of clinical and immunological remission, are given in the Supplementary appendix and detailed elsewhere[23]. The GEMRITUX study was approved by an institutional review board in Paris, France (Comité de Protection des Personnes Ile-de-France XI).

Here, we aimed to characterize lymphocyte populations and cytokines/chemokines in twenty-five consecutive patients out of the 80 patients of the GEMRITUX trial; these patients were enrolled in the study because they lived in the Paris area which made it possible to perform the flow cytometry analysis within 24h after blood collection. Controls were 27 healthy donors (HD) matched for age, including blood donors and volunteers from UMR_S1155 (Hôpital Tenon, Paris, France) who consented to the study.

We then investigated the dynamic changes of lymphocyte subpopulations and cytokines/chemokines in the 25 PMN patients at baseline and 8 days, 3 and 6 months after treatment with rituximab added to supportive therapy (n=16) or with supportive therapy alone (n=9).

Anti-PLA2R and anti-THSD7A antibody measurements

Anti-PLA2R antibodies (total IgG) were assessed using the quantitative ELISA test commercialized by EuroImmune AG (Lübeck, Germany) as previously described[27]. In brief, sera diluted to 1:100 were incubated with PLA2R already coated microplates and detected by incubation with antihuman IgG HRP conjugate. The final concentrations for each sample were calculated from the calibration curve extinction values plotted against the concentration for each calibrator. ELISA cut-off values were established according to manufacturers' protocol and the results were considered as negative for<14 RU/ml and positive for $\geq$ 14 RU/ml. In our laboratory, the calculated intra and inter-assay variations are <4% and <9%, respectively.

Anti-THSD7A antibodies were assessed using an immunofluorescence assay on HEK293 THSD7A-transfected cells kindly provided by Wolfgang Schlumberger (EuroImmune AG). Negativity of THSD7A was defined as absence of detectable antibodies at serum dilution of 1/10. Antibody positivity was defined as positive staining at serum dilutions of 1/10 or higher.

In THSD7A-positive patients, quantitative measurements were performed using different serum dilutions (1/10, 1/100, and 1/1000).

Detection of PLA2R and THSD7A antigen in kidney biopsies

Detection was performed as previously described[53]. Briefly PLA2R and THSD7A were assessed in glomerular deposits by confocal microscopy with rabbit affinity purified specific anti-PLA2R antibodies or anti-THSD7A antibodies (Atlas Antibodies), followed by goat Alexa 488 conjugated anti-rabbit Fab IgG (Molecular Probes).

Flow cytometry analysis and cytokine/chemokine measurements

Blood (20ml) was sampled according to the planned protocol in lithium heparin tubes. Plasma samples were collected, aliquoted and stored at −80°C until analyzed. Flow cytometry analysis was performed according to previously published methods[54] and is described in supplementary appendix.Plasma samples were collected, aliquoted and stored at −80°C until analyzed. Quantitative determination of 26 cytokines/chemokines (GM-CSF, IFN-α2, IFN-γ, IL-1RA, IL1a, IL1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70 , IL-13, IL-15, IL-17a, IL-35, CXCL-10, CCL-2, CCL-3, CCL-4, CCL-2, TNF-α and IL-2RA) was performed using Human Milliplex HCYTOMAG-60 kits (Millipore) in accordance with the manufacturer protocols[54] .

Statistical analysis

Data are presented as mean ± SEM or average (range). Normal distribution of each set of variables was analyzed by D'Agostino & Pearson omnibus normality test. Student's *t*-test for normally distributed data and non-parametric Mann–Whitney were used to compare variables as appropriate. Paired data before and after rituximab treatment were compared using Student's *t*-test for normally distributed data and paired Wilcoxon signed-rank test for non-parametric. Correlation tests were performed using Spearman nonparametric test. A p value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

DISCLOSURES

The GEMRITUX data analysis committee did not include representatives from Hoffmann-La Roche, which provided rituximab for the study. Hoffmann-La Roche was not involved in or

consulted about the GEMRITUX study design, did not review the manuscript, and did not have access to the data or provide any other support for the study.

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Figures legends

Figure 1. Comparison of B-cell subsets, Treg and NK cells in PMN patients and healthy donors. Multicolor flow cytometry analysis of circulating B, T and NK subpopulations in PMN patients (n=25) and in healthy donors (n=27). (A) Subsets of gated CD19+ (total) B cells were identified on the basis of the expression of surface markers as follows. naïve (IgD$^+$CD27$^-$), switched memory (IgD$^-$CD27$^+$), non-switched memory (IgD$^+$CD27$^+$), double negative memory (IgD$^-$CD27$^-$) and expressed as percentage of total B cells; (B) Treg cells were gated in CD4$^+$ T cells, identified as CD25^{hi}CD127$^{lo/-}$Foxp3$^+$cells, and expressed as percentage of total CD4$^+$T cells (left) and counted cells/µl (right); (C) NK cells were identified as CD3$^-$CD56$^+$ cells gated in lymphocytes and expressed as percentage of lymphocytes; CD56^{bright}CD16$^{-/lo}$ cells were gated in NK cells and expressed as percentage of total NK cells. Each box plot represents the median and the 25th and 75th centiles. Error bars represent the smallest and the largest values. Differences with healthy donors were compared using Student's t-test when distribution was normal or with non-parametric unpaired Mann-Whitney U test. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Changes in B cells in PMN patients treated with NIAT-rituximab. Multicolor flow cytometry analysis of circulating B cells presented for each PMN patient from NIAT-rituximab administration to the end of the follow-up. (A) Kinetics of absolute number of B cells over time from baseline (day 0, n=16), to day 8 (n=16), day 90 (n=14), and day 180 (n=14). (B) Outcome of B cell subsets after NIAT-rituximab compared to baseline (day 0 vs day 180). Levels of B cell subpopulations at day 180 were compared to baseline level using a Wilcoxon signed–rank test *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Changes in Treg cells in PMN patients treated with NIAT-rituximab or NIAT only. Treg analysis was performed by flow cytometry. Tregs were gated in CD4$^+$ T cells and were identified as CD25^{hi}CD127$^{lo/-}$Foxp3$^+$cells. A) Data represent changes in CD25^{high}CD127$^-$Foxp3$^+$ among CD4$^+$ T cells (percentages and absolute numbers/µl) in all patients over time from NIAT-rituximab administration (day 0; n=16), to day 8 (n=15), day 90 (n=14) and day 180 (n=14). Data are normalized by baseline values for each patient at the different time points and are represented as fold change. B) Kinetics of Treg (percentages and absolute numbers/µ) in responders (n=8), non-responders (n=8) and patients treated with NIAT alone (n=9). C) Comparison of Treg levels at baseline in responder (n=8) and non-responders (n=8) with healthy donors (n=27). Paired data at baseline and during follow-up

were compared using Student's t-test for normally distributed data and paired Wilcoxon signed-rank test for non-parametric distribution *p<0.05; **p<0.01; ***p<0.001.

Figure 4. Changes in NK cells in PMN patients treated with NIAT-rituximab or NIAT. NK cells analysis was performed by flow cytometry. NK cells were identified as $CD3^-CD56^+$ cells gated in lymphocytes and expressed as percentage of lymphocytes, $CD56^{bright}CD16^{-/lo}$ cells were gated in NK cells and expressed as percentage of total NK cells. A) Data represent changes in NK-cell percentages over time from NIAT-rituximab administration (day 0; n=16) to day 8 (n=16), day 90 (n=14) and day 180 (n=14). B) Data represent changes in $CD56^{bright}CD16^{-/lo}$-cell percentages over the time from NIAT-rituximab administration (day 0; n=15) to day 8 (n=14), day 90 (n=13) and day 180 (n=13). C) and D) show absence of changes for NK and $CD56^{bright}CD16^{-/lo}$ cells in patients treated with NIAT only. Paired data at baseline and during follow-up were compared using Student's t-test for normally distributed data and paired Wilcoxon signed-rank test for non-parametric distribution *p<0.05; **p<0.01; ***p<0.001.

Figure 5. Correlation between Treg changes and B-cell depletion in responder and non-responder patients. Linear regression representation comparing B cells depletion and Treg increase at day 180. Data are expressed as difference between baseline and day 180 values for responder (n=7) and non-responder patients (n=7).

Figure 6. Plasma level of TNF-α in PMN patients treated with NIAT-rituximab. A) Quantitative measurement of TNF-α in the plasma of patients (n=11) and healthy donors (n=9); B) TNF-α levels in patients over time from NIAT-rituximab administration (day 0; n=8), to day 8 (n=7), day 90 (n=6) and day 180 (n=7). Differences with healthy donors were compared using non-parametric unpaired Mann-Whitney U test. Paired data at baseline and during follow-up were compared using Student's t-test *p<0.05; **p<0.01; ***p<0.001

Table 1: Demographic, clinical and biologic characteristics of the study populations

	Patients (N=25)	Healthy Donors (N=27)
Gender (F/M)	5/20	10/17
Age	57 (26-74)	49 (25-66)

	NIAT patients (N=9)	NIAT-rituximab patients (N=16)
Protein/creatinine ratio (mg/g)	7590 (3440-11000)	6250 (3170-15900)
Serum albumin level (g/dl)	2.3 (1.5- 2.8)	1.9 (1.2- 2.9)
Serum creatinine (mg/dl)	1.21 (0.69-2.07)	1.19 (0.68-2.8)
PLA2R-Ab titer (ELISA, RU/ml)*	351.55 (20-538.5)	145.2 (31.2-2900)

Data are expressed as median and range.

* Median and IQR of PLA2R-Ab titer in all patients with PLA2R-Ab

Table 2. Immunopathological data and outcome

Patient Number	PLA2R-Ab				PLA2R-Ag Biopsy	PLA2R-related MN	Rituximab	Clinical remission	Immunological remission
	D0	**D8**	**M3**	**M6**					
1	67.1	NA	0	32.87	Positive	YES	YES	NO	PR
2	0	0	0	0	Negative	NO	YES	NO	-
3	2900	2486	1750	1870	Positive	YES	YES	NO	NO
4	1580	1450	120.5	160.6	Positive	YES	YES	NO	PR
5	36.1	7.5	0	0	Positive	YES	YES	NO	CR
6	216.3	126.1	0	0	Positive	YES	YES	YES	CR
7	1379	1624	49.1	30.1	Positive	YES	YES	YES	PR
8	74.1	NA	0	22.9	Positive	YES	YES	YES	PR
9	1660	1550	137.8	NA	Positive	YES	YES	NO	undetermined
10	16.9	6.9	0	0	Positive	YES	YES	NO	CR
11	31.2	50.7	0	0	Positive	YES	YES	YES	CR
12	0	0	0	0	Negative	NO	YES	NO	-
13	0	0	0	0	Positive	YES	YES	YES	-
14	0	0	0	0	Positive	YES	YES	YES	-
15	0	0	0	0	Positive	YES	YES	YES	-
16#	0	0	0	0	Negative	NO	YES	YES	CR
17	538.5	456.6	995.5	961.1	Positive	YES	NO	NO	NO
18	384.3	206.1	88.8	51.8	Positive	YES	NO	NO	PR
19	369.5	345.5	100.5	699.7	Positive	YES	NO	NO	NO
20	333.6	276.3	84.5	NA	Positive	YES	NO	NO	undetermined
21	0	0	0	0	Negative	NO	NO	NO	-
22	31.2	24.6	16.5	15.1	Positive	YES	NO	NO	PR
23	440.9	NA	278.4	68.5	Positive	YES	NO	NO	PR
24	24.2	NA	0	0	Positive	YES	NO	Yes	CR
25	20.5	18.2	28.7	0	Positive	YES	NO	Yes	CR

 # Patient 16 had anti-THSD7A antibodies

THSD7A-Ab titer was measured using immunofluorescence test. Titers were 1:100 at D0 and D8. Antibodies were then undetectable at M3 and M6. THSD7A antigen was detected in the subepithelial immune deposits.

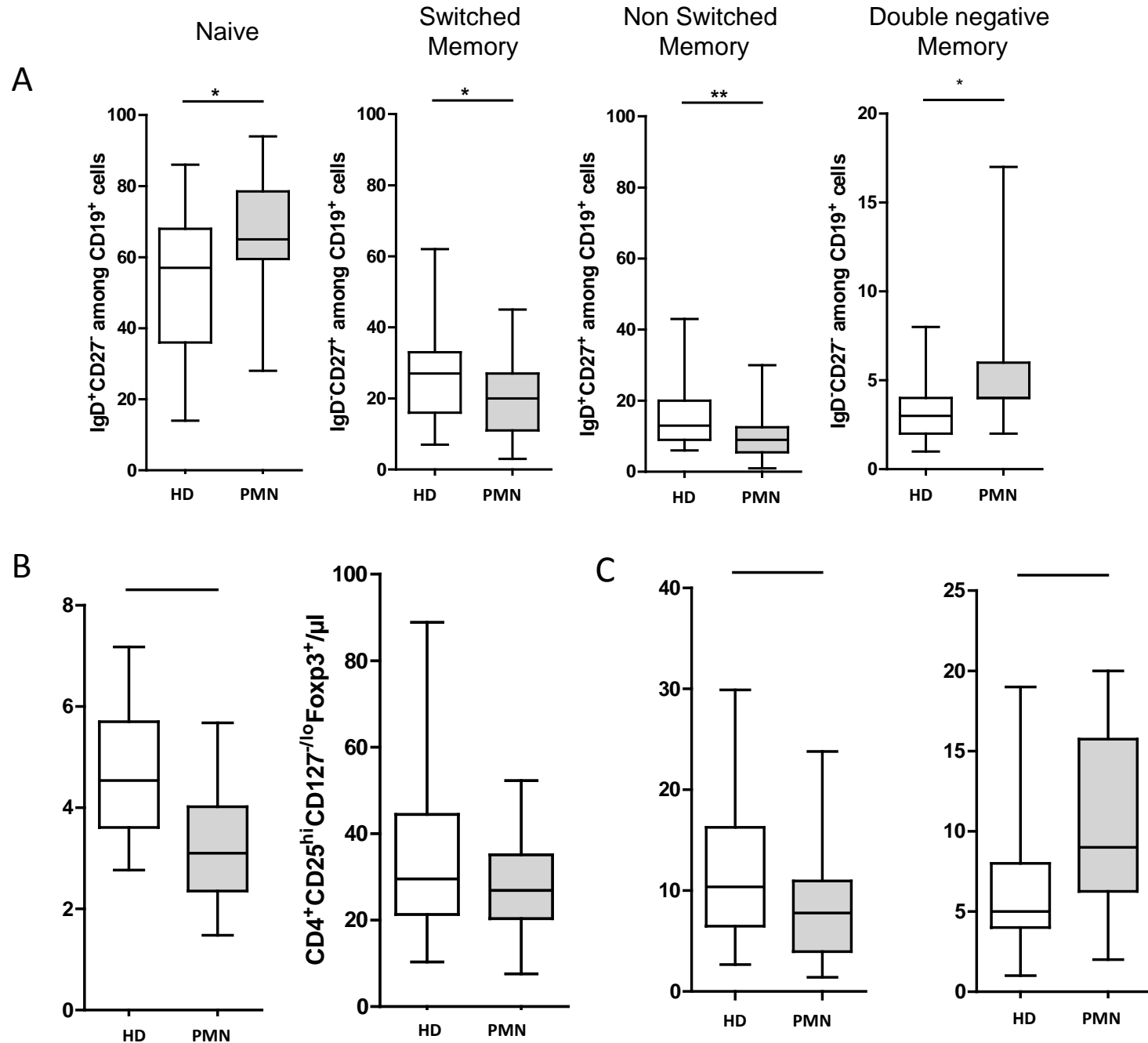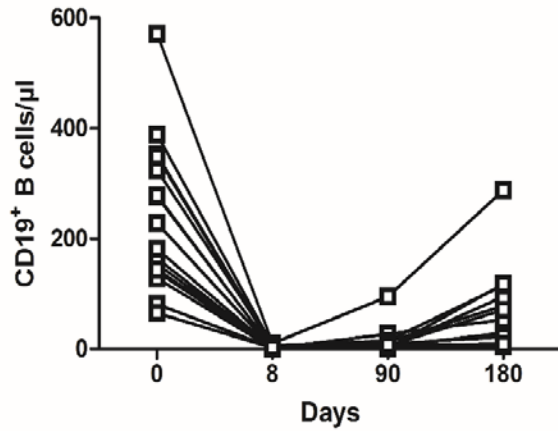# Figure 1: Comparison of B-cell subsets, Treg and NK cells in PMN patients and healthy donors

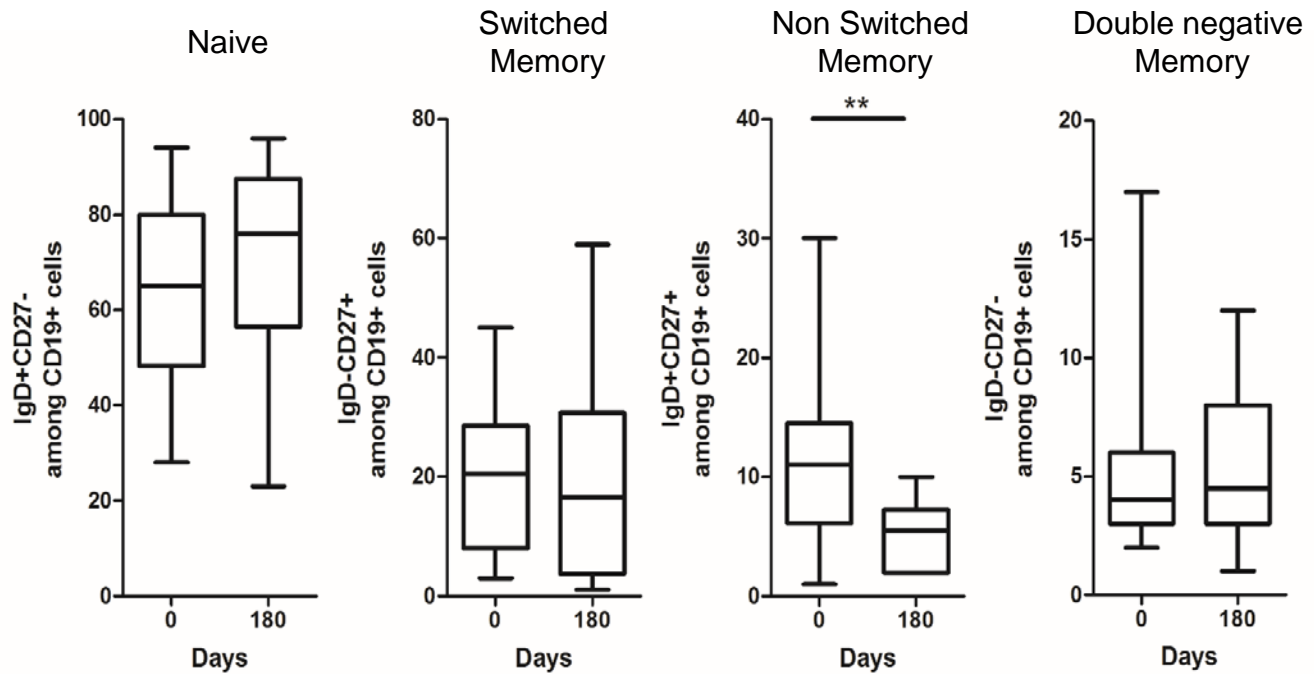**Figure 2: Outcome of B cells in PMN patients treated with NIAT-rituximab**

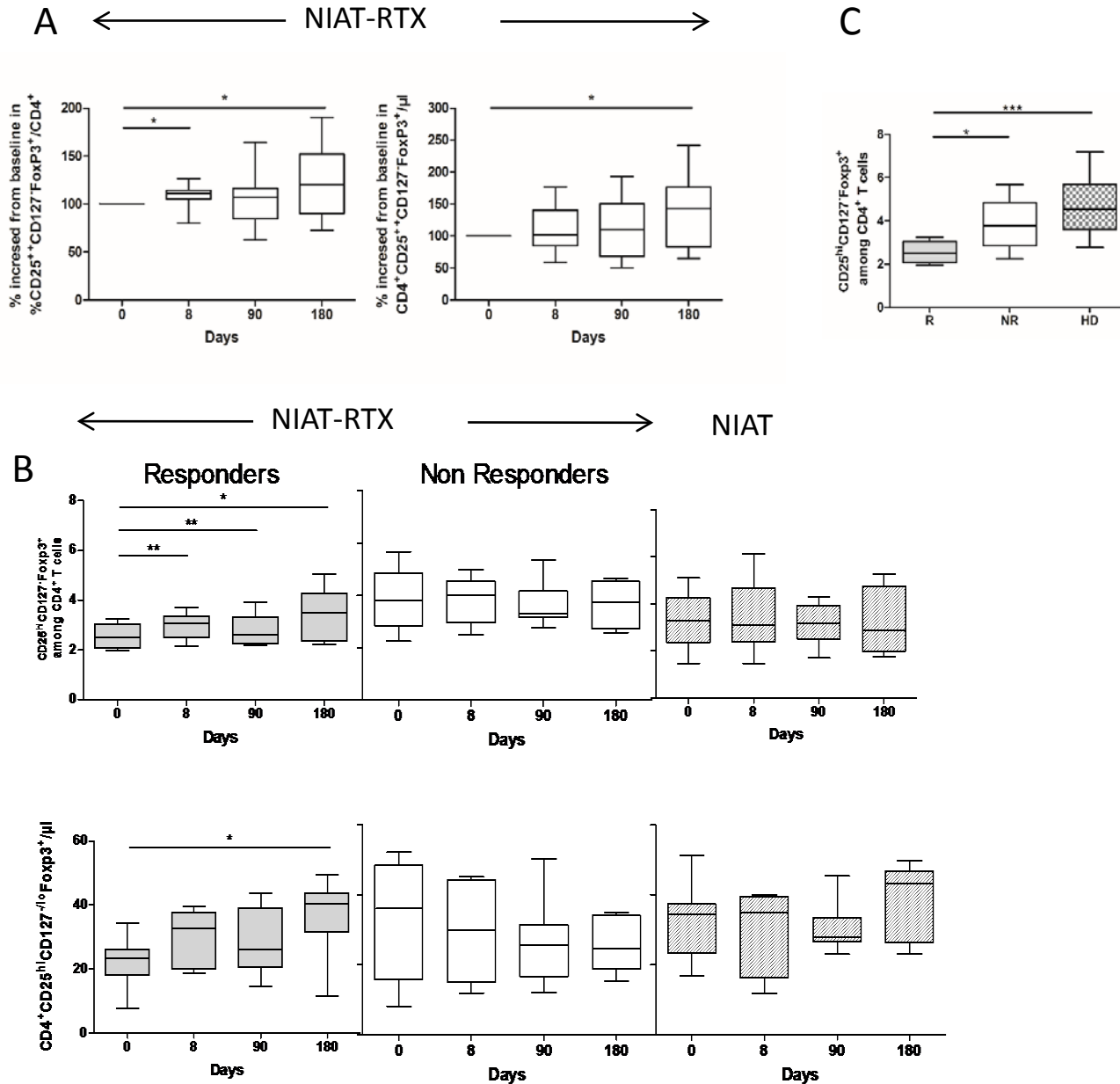**Figure 3:** Changes in Treg cells in PMN patients treated with NIAT-rituximab or NIAT only

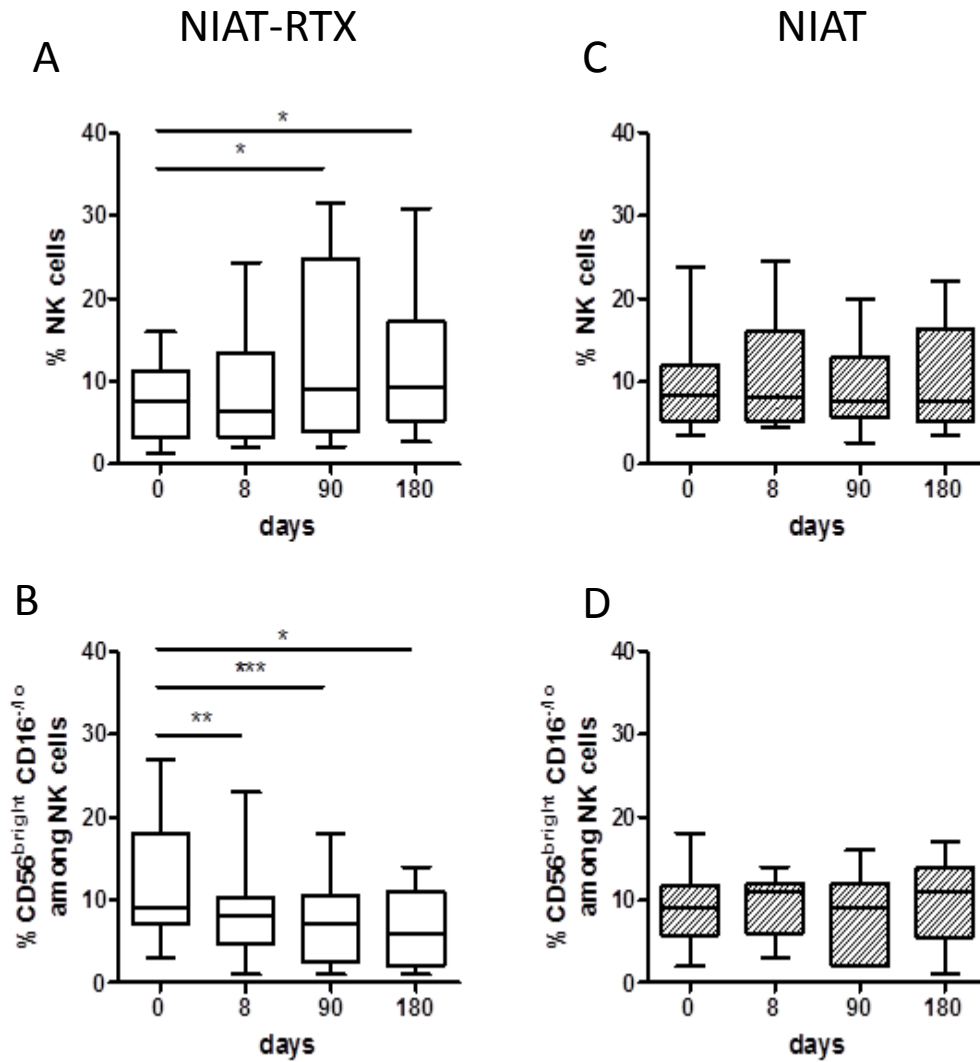**Figure 4: Changes in NK cells in IMN patients treated with NIAT-Rituximab or NIAT only**

Figure 5: Correlation between Treg changes and B-cell depletion in responder and non-responder patients
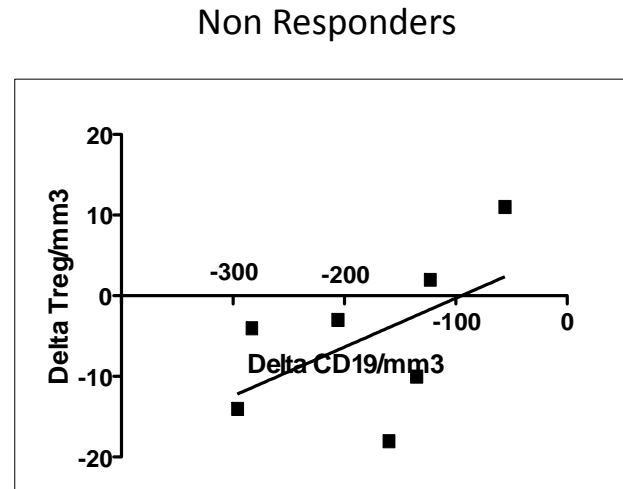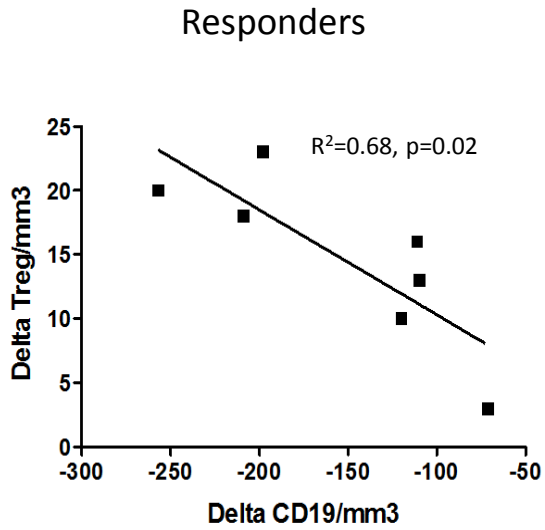
Figure 6: Plasma level of TNF-α in PMN patients treated with NIAT-rituximab