Long-term exposure to monoclonal anti-TNF is associated with an increased risk of lymphoma in BAFF-transgenic mice

Short title: Anti-TNF and risk of lymphoma in BAFF-Tg mice

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Abbreviations:

Ab: antibody

ADA: adalimumab

BAFF: B cell activating factor belonging to the TNF family

ETA: etanercept

IBD : Inflammatory bowel diseases

MTX: methotrexate

NHL: non Hodgkin lymphoma

pSS: primary Sjogren's syndrome

RA: rheumatoid arthritis

RF: rheumatoid factor

SD: standard deviation

SIR: standardized incidence ratio

SLE: systemic lupus erythematosus

Tg: transgenic

TNF: Tumor Necrosis Factor

TNFi: TNF inhibitor

Abstract (197 words)

The impact of treatment on the risk of lymphoma in patients with rheumatoid arthritis (RA) is unclear. Here, we aimed to assess if the risk of lymphoma differs according to the type of Tumor Necrosis factor inhibitor (TNFi), comparing monoclonal anti-TNF antibodies (Ab) to the soluble TNF receptor. We used BAFF-transgenic (Tg) mice as a model of autoimmunity-associated lymphoma. Six-month aged BAFF-Tg mice were treated with TNFi for 12 months. Histological examination of the spleen, assessment of the cellular composition of the spleen by flow cytometry and assessment of B cell clonality were performed at sacrifice. Crude mortality and incidence of lymphoma were significantly higher in mice treated with monoclonal anti-TNF Ab compared to both controls and mice treated with the soluble TNF receptor, even at high dose. Flow cytometry analysis revealed decreased splenic macrophage infiltration in mice treated with monoclonal anti-TNF Ab. Overall, this study demonstrates, for the first time, that a very prolonged treatment with monoclonal anti-TNF Ab increase the risk of lymphoma in B cell-driven autoimmunity. This data suggests a closer monitoring for lymphoma development in patients suffering from B cell-driven autoimmune disease with long-term exposure to monoclonal anti-TNF Ab.

Introduction

Autoimmunity is associated with an increased risk of lymphoma. This risk is well demonstrated in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and primary Sjogren's syndrome (pSS) (1). Two major risk factors have been identified. The first one is the activity of the autoimmune disease, as demonstrated in patients with RA (2) and with pSS (3). The second one is the use of immunosuppressive drugs. It is, indeed, well known that immunosuppression may induce specific types of lymphoma, particularly Epstein-Barr virus -associated lymphomas as described in post-transplant lymphoproliferation disorders (4) or in Crohn's disease treated with thiopurines (5). In SLE patients, the risk of non-Hodgkin lymphoma (NHL) is likely to be associated with exposure to cyclophosphamide and high cumulative steroids (6). In this context, the role of immunosuppressive drugs in promoting lymphoma is difficult to assess since patients that are the most exposed to immunosuppression are also the ones with the most active disease.

Tumor necrosis factor (TNF) inhibitors (TNFi) therapy has revolutionized the management of patients with RA as well as of other autoimmune and inflammatory diseases. It is the cornerstone of treatment of methotrexate (MTX)-resistant RA. Until now, epidemiological studies did not disclose any increased risk of lymphoma in RA patients treated with TNFi (7,8). However, in these studies, the median duration of treatment is approximately 4 years and the potential differential risk according to the type of TNFi remains an ongoing matter of debate. Indeed, there are 2 types of licensed TNFi: a dimeric soluble form of p75/TNF receptor 2 [the TNF-R2-Fc, etanercept (ETA)] and monoclonal anti-TNF antibodies (Ab) [infliximab, adalimumab (ADA), golimumab and certolizumab]. Differences in term of efficacy and safety profile between these 2 types of TNFi are already established. Etanercept is not effective in inflammatory bowel disease (9), and is probably less effective than monoclonal anti-TNF Ab in both uveitis (10) and psoriasis (11). Infectious safety profile also

differs. The risk of opportunistic infections and of reactivation of latent tuberculosis has been shown to be higher with monoclonal anti-TNF Ab than with ETA (12). These data underline the notion of differences in the mechanism of action of TNFi that might differentially impact the risk of lymphoma. In 2010, the French registry Research Axed on Tolerance of Biotherapies (RATIO) found that the risk of lymphoma was higher than the risk found in the general population in patients treated with infliximab or ADA [standardized incidence ratio (SIR) = 3.7 (2.5-5.4)], although remaining in the range of what is expected in patients with long-term active RA. On the other hand, in the same study, the risk of lymphoma in patients treated with ETA reached that of the general population (SIR = 0.9 (0.4-1.8)) (13). In the Japanese RA cohort SafEty of biologics in Clinical Use in Japanese patients with RhEumatoid arthritis (SECURE), patients exposed to infliximab had a significantly higher risk of lymphoma than patients exposed to etanercept with an unadjusted incidence rates of 3.38 (2.57-4.38) with infliximab compared to 1.30 (0.87-1.87) with etanercept ($p< 6.6 \times 10^{-4}$)(14). Of note, in the British Society for Rheumatology Biologics Register for Rheumatoid arthritis (BSRBR-RA) registry, RA patients exposed to etanercept had the lowest crude incidence rate of lymphoma compared to those with infliximab or ADA but the difference did not reach statistical significance after adjustment (15). More recently, it has been demonstrated that patients with inflammatory bowel diseases (IBD) treated with TNFi (only monoclonal anti-TNF Ab) are exposed to an increased risk of lymphoma (16).

To decipher the impact of the different TNFi on the risk of lymphoma associated with autoimmune diseases, we used B cell activating factor belonging to the TNF family (BAFF)-transgenic (Tg) mice, which are a model of lymphoma complicating B cell autoimmunity. These mice develop a B cell-driven autoimmune disease with clinical and biological symptoms reminiscent of RA [rheumatoid factor (RF), polyarthritis], SLE [anti-double strand (ds)DNA, hypergammaglobulinemia, glomerulonephritis, lymphadenopathies, splenomegaly]

and SS (sialadenitis) [16–18]. Three to five percent of BAFF-Tg mice spontaneously develop B cell lymphoproliferative disease during aging. Notably, development of B cell lymphoproliferative diseases in these mice seem to be linked to the action of TNF since introduction of TNF deficiency into BAFF-Tg background (TNF-/- BAFF-Tg mice) leads to a surprisingly high incidence of B cell lymphoma (38% of mice at 12 month of age) (20). To extend these previous findings, we intended to evaluate the risk of lymphoma induced by a long-term treatment with the 2 types of TNFi in these BAFF-Tg autoimmune mice.

Material and methods

Mice

BAFF-Tg mice, with a C57BL/6 genetic background, were provided by F. Mackay (Monash University, Melbourne, Australia) (17). Animals chosen for experimental procedure were homozygous for the BAFF transgene. The genotype of BAFF-Tg mice was determined using PCR on genomic DNA obtained from 5-mm tail snips. Since spontaneous lymphomas have been shown to occur in BAFF-Tg mice aged of 12 -18 months (20), we decided to start treating the mice aged of 6 months for 12 months to be able to assess the impact of TNFi on the risk of lymphoma associated with B cell auto-immunity. Euthanasia was performed after 12 months of treatment or before in case of criteria of imminent death allow the collection of terminal tissue (21).

Treatment of BAFF-Tg mice with TNFi

The number of mice to include in experimental design was calculated based on data reported in BAFF-Tg.TNF$^{-/-}$ mice (20). In this study, 38% of the BAFF-Tg.TNF$^{-/-}$ mice developed lymphoma compared to 5% usually described in the BAFF-Tg mice (17). Given the fact that TNFi treatment would be less potent than TNF$^{-/-}$, we have made the hypothesis of the incidence of 30% of lymphoma in BAFF-Tg mice treated with TNFi compared to 5% in the control group. With a risk alpha of 5%, and a power of 80%, a minimum of 12 mice were needed in each group. Mice were randomized into 4 groups of treatment, as follows:

- anti-mouse TNF monoclonal antibody (clone TN3 19.12 (referred as TN3) + MTX (n=15)
- humanized monoclonal anti-TNF antibody, adalimumab (referred as ADA) + MTX (n=13)

- human recombinant TNF-R2-Fc fragment, etanercept (referred as ETA) + MTX (n=15)
- controls (n=22), consisting of mice treated with water for injection + MTX (n=8) and immunized mice treated with the 3 anti-TNF without MTX leading to undetectable drug levels as soon as week 8 (n=14)

An additional group of BAFF Tg mice was treated with ETA at high dose + MTX (referred as ETA high dose). To prevent immunization against TNFi, MTX (5 mg/kg) was administered intraperitoneally within minutes before the first TNFi injection and repeated only twice at 24 and 48 hours as previously described (22).

All the drugs were administrated by intraperitoneal (IP) injection. Details of the doses and frequency of administration of the drugs are presented in the Supplemental Table 3.

TNFi drug monitoring and anti-drug antibody (ADAb) detection

Blood samples were drawn every 4 weeks starting at week 8 of treatment. Serum drug concentration (ADA and ETA) and detection of anti-drug Ab were simultaneously measured using the Lisa Tracker® (Theradiag) duo enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction [17]. An in-house sandwich ELISA was used to measure serum TN3 drug concentration.

Lymphoma assessment

Spleens were divided into three parts: one for flow cytometry analysis (single cell suspension), one for clonality assessment and one for histological assessment. Since BAFF-Tg mice are characterized by splenomegaly and increased in B cell compartment, assessment of the presence of a B cell lymphoma versus B lymphoid hyperplasia is difficult. Thus, to detect lymphoma with high specificity in our mice, we used the three following parameters: 1-

presence of tumor-like masses clinically assessed (lymphadenopathies, tumoral spleen); 2-histological examination of hematoxylin and eosin slides based on a specific composite score we have developed (see supplementary methods) in the absence of a pre-existing one; and 3-assessment of the clonality (see supplementary methods). Diagnosis of lymphoma was made when 2 of these 3 criteria were present.

Ethics approval and consent to participate: This study was approved under number by the regional animal care and ethics committee (CEEA34.CN.089.12.). Animal care and use was in accordance with the EU Directive 2010/63/EEU. The institute was approved as compliant with ETS123 recommendations for animal breeding and with Standards for Human Care and Use of Laboratory Animals.

Statistical analysis

All analyses were performed using GraphPad Prism 7 software (Version 7.04). Continuous variables were summarised as mean (standard deviation) (SD). Non-parametric tests were used to compare difference in continuous variables between groups (two groups: Mann Whitney U test; > 2 groups: Kruskal-Wallis test followed by Dunn's multiple comparison test). Categorical variables were presented as frequencies (%), and difference in proportions were analysed using Fisher's exact tests. Crude mortality and progression free survival (PFS) without lymphoma were compared among the different groups using log rank test.

Results

Immunogenicity and pharmacokinetics of TNFi in BAFF-tg mice

B cell lymphomagenesis linked to activity of autoimmune diseases or to immunosuppression are supposed to be a long-term process. Thus, to test the impact of TNFi on the risk of lymphoma in BAFF-Tg mice, we performed chronic exposure to the drugs for one year (half of the life of mice). However, treatment of mice with TNFi, murine or humanized, lead to immunization occurring as soon as 2 weeks after starting the treatment (23). To prevent such immunization, we performed a short course of intraperitoneal administration of MTX at a dose of 5 mg/kg at day 0, 1 and 2 as previously reported (22). This led to a complete prevention of immunization and to the maintenance of significant drug concentration during the 12 months of the experiment, as we previously published (22) . Taking advantage of this strategy, we obtained the following mean +/- SD serum TNFi concentrations over 1 year: anti-mouse TNF monoclonal antibody (clone TN3) referred as TN3 (69 +/- 50 µg/ml), ADA (105 +/- 67 µg/ml) and ETA (7 +/- 9 µg/ml). While serum concentrations of TNFi differed among these three groups, reaching higher concentration of ADA was an objective in our study design given its lowest capacity to inhibit murine TNF compared to ETA (24). This observation was confirmed by performing a cytotoxicity assay using TNF-sensitive L929 cells as targets. Inhibitory concentration leading to 50% decrease in TNF-induced L929 cell death was 300 µg/ml and 25 µg/ml for ADA and ETA respectively (Figure 1.A). To assess the bioactivity of the different anti-TNF in vivo, we performed the L929 assay using the sera from the mice treated by anti-TNF (ADA, n=4; ETA, n= 4 or untreated mouse as a control) and confirmed the bioactivity of the two types of TNF blockers used in the mice (Figure 1.B).

BAFF Tg mice treated with monoclonal anti-TNF have a reduced overall survival rate

Overall survival rate of the four groups of mice was analyzed during the 12 months of treatment with TNFi. The median [range] duration of survival was 221 [56-374] days in the TN3 group, 269 [10-374] days in the ADA group, 368 [119-374] days in the ETA group and 374 [212-374] days in the control group. We observed a significantly reduced survival rate for the BAFF-Tg mice treated with the two monoclonal anti-TNF Ab compared to the control group (Figure 2). Conversely, survival of mice treated with ETA was not significantly different from controls. Of note, among the controls, no difference was observed between immunized mice without detectable drug levels and mice treated with water + MTX (supplemental table 1).

Exposure to the different types of TNFi does not impact manifestations of auto-immunity in BAFF Tg mice

We, then, assessed if signs of autoimmunity differed in the different groups of mice (ADA, ETA, controls) and thus might explain the difference in term of overall survival. We did not observe any difference between groups regarding the types of the B cells infiltrating the spleen except for T1 and T2 that were increased in mice treated with ADA compared to controls and without difference between ETA and ADA groups (Figure 3 A-D). There was no difference concerning the titer of auto- Ab (anti-DNA and RF), the IgM and IgG serum level (Figures 3 E-H), the renal involvement by studying the presence of glomerular (Figure 3I) and tubule-interstitial involvement (Figure 3J). All together, we were unable to detect any impact of the different TNFi treatments on the signs of autoimmunity in the BAFF-Tg mice.

Chronic exposure and high dose of monoclonal anti-TNF Ab are associated with an increased prevalence of lymphoma in BAFF Tg mice

Based on our stringent definition of lymphoma (see methods: presence of 2 criteria among macroscopic abnormality, histologic composite score and assessment of B cell clonality), we

observed a statistically significantly higher incidence of lymphoma in the BAFF-Tg mice treated by monoclonal anti-TNF Ab compared to controls (table 1). For more details concerning TNFi-related B cell lymphomas see supplemental table 2. Interestingly, conversely to monoclonal anti-TNF, etanercept is able to inhibit lymphotoxin-α (LTα) that is known to participate to germinal center formation. Thus, we looked at the size of the follicular compartment between the different groups of mice and did not detect a significant difference in this specific item of the histologic composite score (supplemental Figure S1). This suggests that the difference in term of lymphoma incidence was not linked to a protective effect of etanercept via an inhibition of LT. Since no mouse presented lymph node enlargement without splenomegaly, histologic examination and assessment of B cell clonality were performed only on spleens. Assessment of survival rate without lymphoma confirmed the higher incidence of lymphoma in mice treated with monoclonal anti-TNF Ab (Figure 2).

Notwithstanding that our objective was to reach a 10-fold higher concentration of ADA than ETA (because of the substantial lower efficacy of ADA than ETA on murine TNF), we decided to test if treatment with higher concentration of ETA might modify the occurrence of lymphoma. This new group of mice was treated for 12 months at a concentration of 40 mg/kg (supplemental table 1) three times a week. Obtained mean concentration of ETA +/- SD was 84.5 +/- 67 µg/ml. Only one lymphoma was observed among the 11 mice treated with high ETA dosage, leading to similar low incidence of lymphoma (9%) observed in control mice. Thus, the differences of drug concentrations did not explain the observed difference in incidence of lymphoma in these mice.

Chronic exposure to monoclonal anti-TNF Ab is associated with a decreased macrophage infiltration

To get more insights into the mechanisms associate with different lymphomagenesis incidence associated with TNFi administration in mice, we explored the effect of the tested TNFi on the local microenvironment surrounding lymphoma. We particularly focused our attention on three cellular subsets known to be strongly involved in lymphoma immunosurveillance and which express membrane TNF i.e. Natural killer cells (NK), Foxp3+ regulatory T cells (Treg) and macrophages. Unexpectedly, we did not detect any significant splenic NK-cell infiltration in these mice (data not shown). In addition, we did not detect any significant difference between splenic pool of Treg between groups (Figure 5A). Conversely, flow cytometry analysis using an exclusion panel revealed that splenic macrophage infiltration was significantly reduced in the ADA group compared to ETA (Figure 5B). The decrease in macrophage infiltration in the ADA group compared to ETA was confirmed in IHC using CD68 staining (Figure 5C-E). We aimed to further assess the origin of this observed decrease splenic macrophages infiltration in mice treated with ADA. We did not detect an impact of *in vivo* exposure to TNFi on the capacity of migration of monocytes in response to MCP-1/CCL2 stimulation (Supplemental Figure 1).

Finally, we investigated the nature of the macrophages infiltrating the spleen. We measured INOS in as a M1-like marker and MARCO as a M2-like marker(25) by qPCR. Our results showed that M1-like were more decreased with ADA than with ETA and that M2-like were spared by ADA but not by ETA (Figure 5 F-G).

Discussion

In this study, we showed that long-term exposure to monoclonal anti-TNF Ab but not to the soluble TNFR2-Ig in BAFF-Tg mice, a mouse model of B cell autoimmunity, predisposed to develop lymphoma. We found that this higher risk of lymphoma in mice treated with monoclonal anti-TNF Ab compared with ETA could be linked in part to a decrease in macrophage infiltration of lymphoid organ that might be involved in lymphoma immunosurveillance.

Difference in term of survival between BAFF-Tg mice treated or not by TNFi could have been linked to a negative impact of TNFi on autoimmunity. Actually, TNFi have been shown to be inefficient in the two major autoimmune diseases driven by BAFF i.e. SLE (26) and SS (27). Moreover, several studies support an aberrant immune-regulatory effects of TNF in SLE (28). Interferon (IFN) signature is well established in SLE and in SS and a cross regulation between TNF and IFN type I has been described. It has been demonstrated that TNF regulates generation of plasmacytoid dendritic cells (pDC), the main producers of IFN-α and IFN-α release by pDC (29). In patients, occurrence of auto-Ab to nuclear antigens as well as occasional, transient lupus-like syndromes in patients exposed to TNFi might suggest a protective role of TNF in SLE (30). However, this hypothesis remains controversial. Some reports including open label studies have suggested that TNFi might be efficient in some SLE patients (31). In the present study, we did not observe any impact of TNFi treatment on manifestations of autoimmunity confirming this balanced effect, and further suggesting that the observed increased mortality in mice treated by TNFi was not linked to flare of the autoimmune disease.

We observed an increased incidence of lymphoma in BAFF-Tg mice treated by monoclonal anti-TNF Ab. We paid great attention to the definition of lymphoma given the basal B-cell hyperactivity observed in BAFF-Tg mice (17). Batten *et al.* defined the presence of lymphoproliferation by macroscopic abnormalities and histological examination (20). In our study, we also added assessment of B cell clonality to be more stringent. However, given the strict definition of lymphoma we had (2 out of 3 items among macroscopic tumor, histological score and clonality), lymphoma incidence might have been misestimated. Nevertheless, the percentage of lymphoma observed in these experiments was in line with previously described rates in both BAFF-Tg and BAFF-Tg.TNF$^{-/-}$ mice(20).

As mentioned above, our data suggests that in the context of chronic autoimmune B cell stimulation, long-term inhibition of TNF by monoclonal anti-TNF Ab could increase the risk of lymphoma. Even if the impact of inhibition of TNF on the risk of lymphoma has already been demonstrated by the study of Batten et al(20), this previous model was extreme since these mice were totally deficient for. Here, we demonstrate that inhibition by therapeutic Ab targeting TNF could have the same effect. Two parameters need to be considered before assessing the mechanism leading to lymphomagenesis in these mice. Firstly, mice were treated for 12 months which represents half-life of the mice, hence a very long-term exposure that has no parallel in human. Secondly, the mean blood concentration of ADA was very high (105 g/ml) while that of ETA was 7g/ml. It was planned in our design to have a 10-fold higher concentration of ADA based on the lower efficacy of ADA than ETA to bind mouse TNF (Figure 1). However, while we also used a higher dose of ETA that did not increase the risk of lymphoma, we cannot exclude that high doses of ADA might have promoted lymphoma in addition to a differential mechanism of action between soluble TNF-R2-Fc and monoclonal anti-TNF Ab. Some data from human studies suggest that the risk of lymphoma could

increase with the dose of TNFi Ab. In placebo-controlled phase and long-term extension studies assessing efficacy of golimumab, patients treated with higher dose of golimumab (100 mg monthly) were likely to be at greater risk of lymphoma than is expected in patients with RA (SIR= 6.69 [2.45–14.56]) (32). At a classical dosage of 50-mg monthly, the SIR was 1.71 (0.04–9.55). This suggests a possible dose effect of TNFi regarding the risk of lymphoma and it highlights the necessity in clinical practice of step-down strategies in patients in remission or low disease activity.

If the differential risk of lymphoma between TNFi drugs is not associated with their dose, it could be due to a different mechanism of action between TNFi. From our results, one could speculate the presence of 1- a deleterious impact of monoclonal anti-TNF Ab on lymphoma immunosurveillance; and/or 2- a protective role of ETA. This second hypothesis could be linked to the inhibition of LTα by ETA but not by monoclonal anti-TNF Ab (33). It is well demonstrated that in mice, LTα is critical for the development and maintenance of splenic and lymph node microarchitecture (34). In addition, LTα participates to the formation of germinal centers (GC) (35) and to generation of memory B cells (36). Last, it has been demonstrated that LTα participates to the establishment of a permissive niche for lymphoma(37). However, in our study, we did not observe a difference in term of size of the follicular compartment between the three groups of treatments suggesting that ETA did not inhibit GC formation in these BAFF Tg mice. In addition, treatment with ETA did not decrease the incidence of lymphoma compared to the control group even at higher dose.

Immune surveillance plays an important role in B-cell lymphomagenesis as demonstrated by development of immune checkpoint inhibitors in the treatment of Hodgkin lymphoma and NHL (38,39). Clearly, several cellular actors of immune surveillance might be affected by TNFi. Firstly, we previously found that NK cells were negatively impacted by TNFi but

without any difference between ETA and monoclonal anti-TNF Ab (40). In the current study, we did not detect any significant NK cells infiltrate within the spleen of BAFF-Tg mice. Secondly, Treg might be involved. The impact of Treg in the microenvironment of lymphoma is controversial (41). Some studies suggest that the presence of Treg within the microenvironment could be associated with better prognosis (42). Conversely, other studies suggest that Treg through a strong immunosuppressive effect on anti-tumor response are major deleterious factor for tumor control (43). Interestingly, recent report demonstrated that ADA but not ETA was able to induce expansion of Treg through the membrane TNF-TNFR2 pathway (44). However, in our study we did not detect any difference in splenic Treg infiltration in mice treated with ADA, ETA or controls. Thirdly, the role of macrophages in anti-lymphoma immunosurveillance is now strongly established (45). Very recently, the development of an anti-CD47 immunotherapy aiming to reverse inhibition of macrophages has shown very promising results in NHL (46). This highlights the role of macrophages in controlling lymphoma. Here, we found a decreased infiltration of the spleen by macrophages after exposure to anti-TNF inhibitors with data suggesting that M1-like were more decreased with ADA than with ETA and that ADA spared M2-like conversely to ETA. This could be deleterious in the context of cancer control. Interestingly, it has been demonstrated that adalimumab could promote M2-like macrophages while ETA could not. The mechanism leading to this effect remains unclear and could involve interaction between the anti-TNF Fc region and Fcγ receptors(47). An acknowledged difference in the mode of action between monoclonal anti-TNF Ab and ETA is a better stability of monoclonal anti-TNF/membrane TNF complexes, possibly leading to reverse signaling and modulating macrophages (48). It may explain the differential effect in IBD patients and the differential risk of latent tuberculosis reactivation observed with different TNFi. Beside macrophages, other actors of

immunosurveillance including cytotoxic T CD8+ could be modulate by anti-TNF and may also play a role.

Collectively, our study demonstrates for the first time that that very long-term inhibition of TNF especially with monoclonal anti-TNF Ab, in the context of chronic autoimmune B cell stimulation, increases the risk of lymphoma in mice. This increase in lymphoma incidence might be driven by a specific mechanism of action of monoclonal anti-TNF Ab for decreasing immune-surveillance by macrophages both quantitatively and qualitatively. Further research aiming to better understand how TNFi impair the phenotype and function of macrophages involved in immune surveillance are needed. This data suggests the need for a closer monitoring of lymphoma occurrence in patients with B cell-driven autoimmune disease with long-term exposure to monoclonal anti-TNFi. This data also underlines the need to decrease the dose of TNFi when the target of treatment is achieved (i.e. remission or low disease activity), as advised in the EULAR recommendations for treatment of RA patients (49).

Declarations

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Table 1: Incidence of lymphoma in the 4 groups of mice

	Mice (n)	Lymphoma (n (%))
TN3	15	5 (33)
ETA	15	0 (0)
ADA	12	4 (33)
Controls	22	1 (5)

Frequencies (percentage) are presented. ADA: adalimumab; ETA: etanercept; TN3: anti-mouse TNF monoclonal antibody

Figures legends

Figure 1: L929 TNF cytotoxicity assay using ADA and ETA. A. Comparison of the concentrations of ETA and ADA allowing rescue of L929 cell lines exposed to murine TNF. Results are mean and SD of 5 experiments. B. L929 assay using serum from untreated mouse (control), sera from mice treated with ETA (n=4) and sera from mice treated with ADA (n=4) to inhibit TNF. Results are shown as mean and SD.

Figure 2: Survival rate of BAFF-Tg mice is significantly reduced in the 2 monoclonal anti-TNF Ab groups compared to controls. Representation of the Kaplan-Meier curve of survival is shown. Mice were randomized into 4 groups of treatment: anti-mouse TNF monoclonal antibody (clone TN3 19.12 (referred as TN3) + MTX (n=15), humanized monoclonal anti-TNF antibody, adalimumab (referred as ADA) + MTX (n=13), human recombinant TNF-R2-Fc fragment, etanercept (referred as ETA) + MTX (n=15) and controls (n=22). Survival is compared using log-rank (Mantel Cox) test.

Figure 3: Assessment of autoimmunity in the BAFF-Tg mice treated with TNFi. Assessment of the cellular composition of the spleen by flow cytometry performed at sacrifice in 10 mice treated with control, 14 treated with ETA and 11 mice treated with ADA and focused on (A) Marginal zone (MZ) B cells, (B) Follicular (Fo) B cells, (C) T1 B cells, and (D) T2 B cells. (E) Anti-dsDNA Abs, (F) RF titers, and IgM (G) and IgG (H) concentrations measured in the sera of the mice at sacrifice (n=18 in the control group, n=12 in group treated with ETA, n=6 in the group treated with ADA and n=12 in the group treated with TN3). Assessment of renal involvement in 19 mice from the control group, 11 mice treated with ETA, 9 treated with ADA and 9 treated with TN3: proportion and severity of glomerular (I)

603 and tubule-interstitial (J) lesions in mice. Results are shown as mean and SD. Mann-Whitney
604 U test. *: $p<0.05$, **: $p<0.01$

605

606 **Figure 4: Survival rate without lymphoma of BAFF-Tg mice treated with TNFi.**
607 Representation of the Kaplan-Meier curve of survival. Mice were randomized into 4 groups of
608 treatment: anti-mouse TNF monoclonal antibody (clone TN3 19.12 (referred as TN3) + MTX
609 (n=15), humanized monoclonal anti-TNF antibody, adalimumab (referred as ADA) + MTX
610 (n=13), human recombinant TNF-R2-Fc fragment, etanercept (referred as ETA) + MTX
611 (n=15) and controls (n=22). Survival rate is compared using log-rank (Mantel Cox) test.

612

613 **Figure 5: Assessment of cellular infiltrate involved in lymphoma immunosurveillance in**
614 **BAFF-Tg mice treated with TNFi.** Flow cytometry analysis was performed on spleen of
615 BAFF-Tg mice treated by control (n=9), ETA (n=8) or ADA (n=6). (A) Splenic Treg cells
616 and (B) macrophages were assessed. (C) Macrophages infiltrate was also assessed by CD68
617 staining in IHC on spleen sections (n=5 for each group). Panels (D) and (E) are representative
618 of CD68 staining in IHC, with x 40 magnification in mice treated with (D) ETA and (E)
619 ADA**.** INOS (F) and MARCO (G) in ADA (n = 6), ETA (n = 6) or controls Ig mice (n = 6)
620 quantified by qPCR. Results are shown as mean and SD. Mann-Whitney U test. *: $p<0.05$, **:
621 $p<0.01$.
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Figure 1

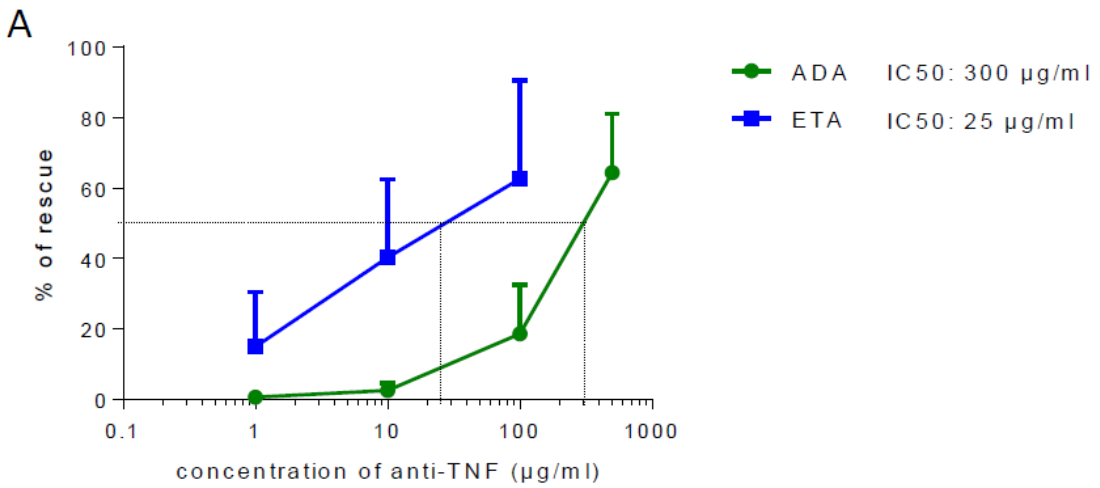

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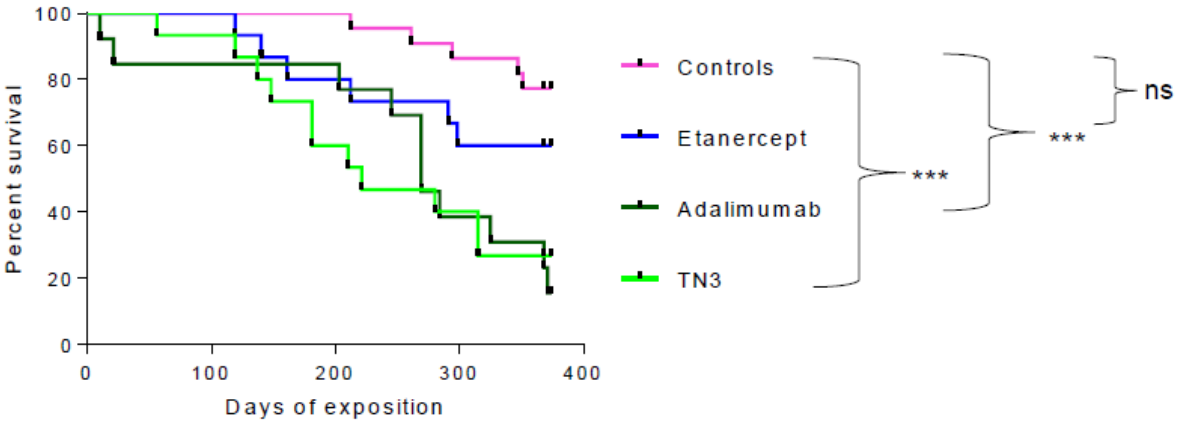
Figure 2



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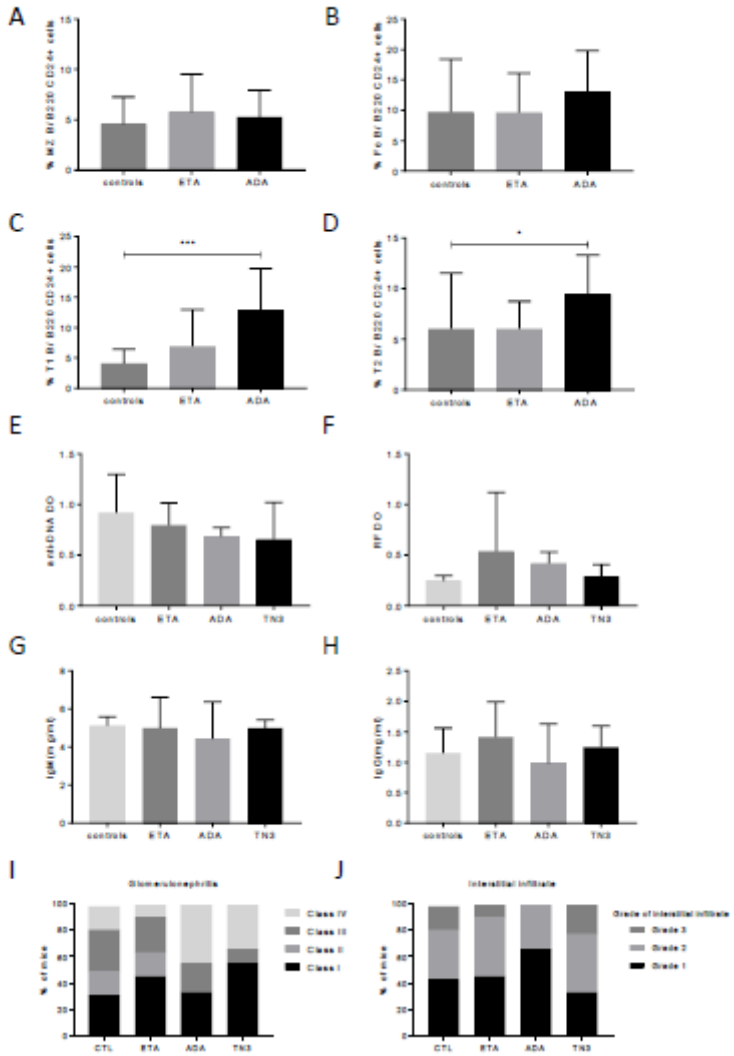

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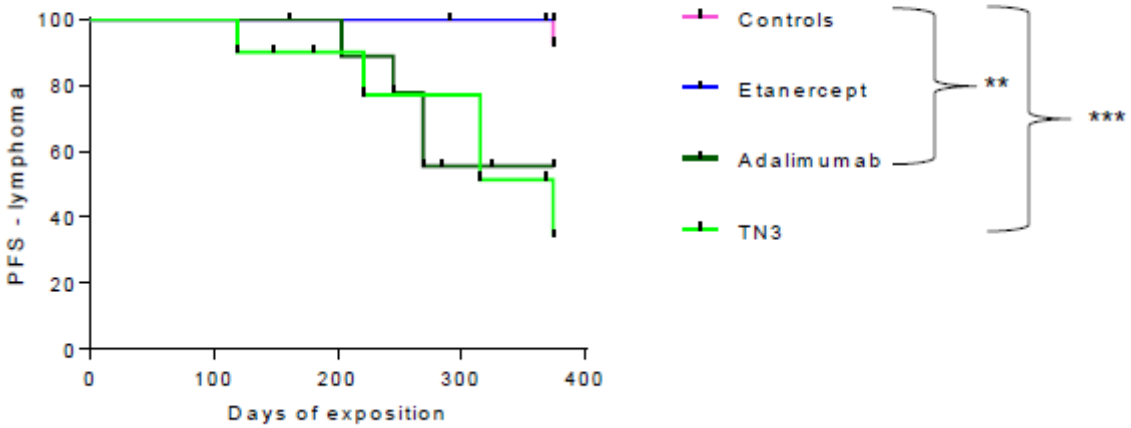
Figure 4



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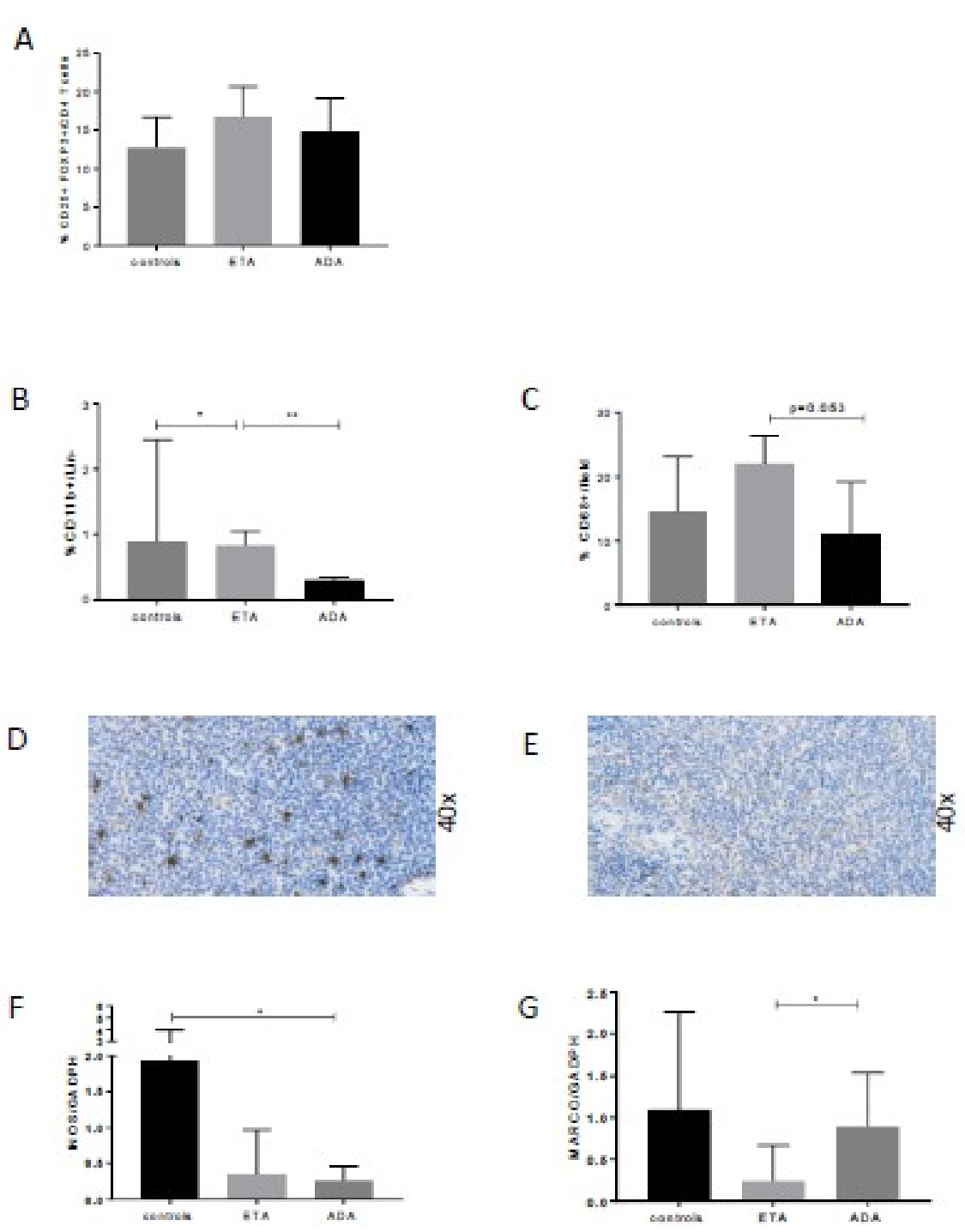
631 Figure 5



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